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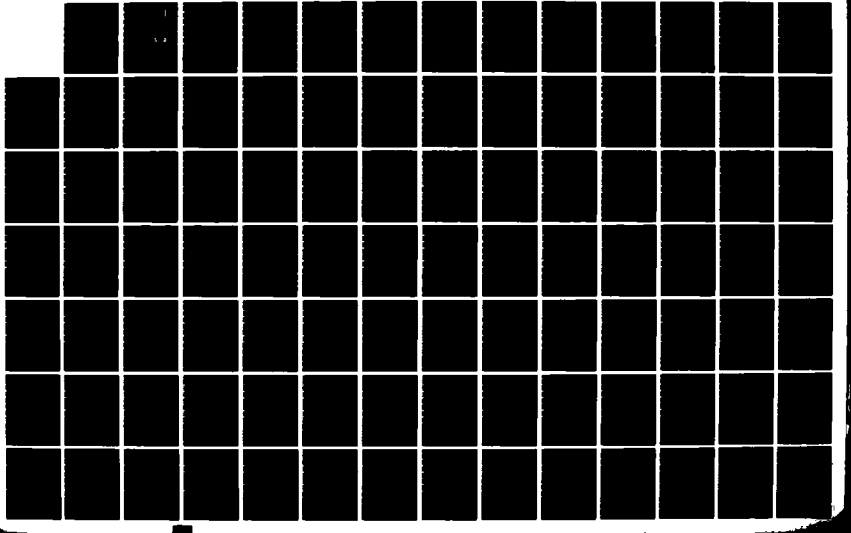

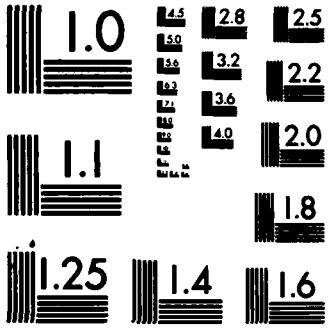

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ANNUAL PROGRESS REPORT

Robert E. Shope, M.D.

January 1980

Supported by

U.S. Army Research and Development Command, Fort Detrick,
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Yale University School of Medicine
New Haven, Connecticut 06510

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Two major findings relating to virus taxonomy were 1) the Nairovirus Supergroup of Bunyavirus-like agents including groups Congo-Crimean hemorrhagic fever, Nairobi sheep disease, Dera Ghazi Khan, Qallyub, Hughes, and possibly Sakhalin, and 2) the serologic relationship of Rift Valley fever virus to the phlebotomus fever group.

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Serologic surveys. A survey of residents of China, mostly of the Peking area showed a very high prevalence of group B, probably Japanese encephalitis reactivity. A high percentage of Liberian human sera had group B and group A (probably chikungunya) reacitivity.

Diagnosis of disease. The first case of disease associated with Pichinde infection in a laboratory worker was diagnosed serologically. Ross River infection was recognized in Canadian tourists returning from Fiji. A focus of LaCrosse encephalitis infection was studied north of New York City.

Development of techniques and models. Further development of the ELISA and the spot-slide FA techniques now permit rapid, sensitive diagnosis of LaCrosse encephalitis, Congo-Crimean hemorrhagic fever, yellow fever and several other arbovirus diseases. Polyacrylamide gel electrophoresis was refined in the study of RNA and proteins of orbiviruses. The Rhipicephalus appendiculatus tick cell line was adapted to trypsinization and mass culture. Purification of arboviral immunoglobulins by immunosorbent chromatography and use of the microcarrier cell culture system for enhanced virus yield were developed. A technique for feeding ticks on capillary tubes was elaborated. Pathogenicity of phlebotomus fever group viruses in cell culture and in mice was determined in anticipation of studies with reassortant viruses of this group. C6/36 Aedes albopictus cell persistently infected with Japanese encephalitis virus served to yield high titered antigen and virus on a continuous basis.

Distribution of reagents. The reference center distributed 518 ampoules of reference sera, viruses, and antigens during 1979, as well as mosquito and vertebrate cell lines.

SUMMARY

Two major findings relating to virus taxonomy were 1) the Nairovirus Supergroup of Bunyavirus-like agents including groups Congo-Crimean hemorrhagic fever, Nairobi sheep disease, Dera Ghazi Khan, Qalyub, Hughes, and possibly Sakhalin, and 2) the serologic relationship of Rift Valley fever virus to the phlebotomus fever grop.

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FOREWARD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Figure | Page

BODY OF REPORT

I. Virus Taxonomy

Nairovirus supergroup: a new antigenic supergroup that includes the CCHF and Nairobi sheep disease groups (J. Casals and G.H. Tignor). A distant relationahip was recently reported between Nairobi sheep disease (NSD) and CCHF and Hazara viruses, which was detected by means of the immunofluorescence (IF) and indirect hemagglutination tests but not by neutralization test (Davies et al., J. Comp. Path., 88: 519, 1978). An extension of these studies to other arboviruses has shown heretofore unreported antigenic relationships among viruses some--Congo, Dugbe, Ganjam, Hazara and NSD--currently placed in the family Bunyaviridae, subdivision "Bunyavirus-like" of the classification scheme established by the International Committee on Taxonomy of Viruses, ICTV (Porterfield et al., Intervirology, 6: 13, 1975-76) and others listed as unclassified (Berge, Arbovirus Catalogue, 1975). Thus far, these relationships have involved only tick-associated viruses.

Until now, the complement-fixation (CF) test has been the technique generally used for the antigenic classification of tick-borne viruses excluding the flaviviruses (Casals, Proceedings of a Symposium, Bratislava, 1971). Usually, a CF screening is done by testing an anti-serum for the problem virus against antigens from all available tick-borne viruses; cross-reactions thus detected were subsequently investigated by neutralization test and, if antigens could be produced, by hemagglutination-inhibition (HI) test.

Initially planned as an investigation of the antigenic relationships of CCHF virus the studies summarized here have been extended to other tick-borne viruses in various antigenic groups as well as ungrouped ones; in addition, and mainly as controls for specificity in the tests, insect-associated viruses from several taxons and antigenic groups have been included. As of now, 54 different serotypes have been represented; in addition to the viruses named in Table 1 and 2 , the following were included: Batai, Bhanja, Bwamba, California encephalitis, Caraparu, Chenuda, chikungunya, Colorado tick fever, dengue 3, Dhori, epizootic hemorrhagic disease of deer, Grand Arbaud, Huacho, Inkoo, Japanese encephalitis, Johnston Atoll, JosCow, Kaisodi, Karimabad, Kemerovo, Lanjan, Lonestar, Manawa, Mono Lake, Oropouche, phlebotomus fever Naples, Sakhalin, Silverwater, Thogoto, Tuyleniy, Upolu, Uukuniemi, VEE, Wad Medani and West Nile.

The techniques used in this study were: HI, when agglutinating antigens could be prepared; indirect IF with spot-slides prepared with cell cultures infected with the viruses; and the neutralization test. The latter when done with CCHF virus was: either a mouse intracerebral test inoculating a mixture of undiluted serum and a constant amount of virus, equivalent to 100 LD_{50}, to groups of 16 mice between 22 and 28 days old; or a kinetic reduction of fluorescent foci of infection (RFFI) (Tignor et al., 1980, Am.J.Trop. Med.& H, in press). Neutralization tests with Bandia virus were by the intraperitoneal route of inoculation to newborn mice, using mixtures of constant amount of virus and serum

dilutions, beginning with undiluted serum. The sera were extracted with acetone and ethyl ether for all the tests; for HI they were in addition adsorbed with goose erythrocytes. The sera were used in serial two-fold dilutions beginning at 1:5 or 1:10 in the HI test, and beginning with undiluted serum in the IF and RFFI tests.

Tables 1 and 2 summarize the current status of the study. HI tests with CCHF, Hazara, Dugbe and Abu Minah antigens, the only ones that we have thus far been able to prepare, have been the key to uncovering a relationship that includes viruses in the groups CCHF, NSD, DGK (Dera Ghazi Khan), Qalyub, Hughes and possibly Sakhalin; no sera for viruses outside these antigenic groups have shown cross-reactions with the above antigens, even at dilution 1:5. In so far as the observations have gone, the neutralization test with CCHF virus, either the mouse test or the RFFI, has shown cross-reactions, to a lesser extent, only among viruses of the same groups, particularly with NSD and Qalyub. No cross-reactions have been seen in neutralization tests with Bandia virus. Cross-reactions have been detected by IF between viruses of the CCHF and NSD groups; to a much lesser degree and only sporadically with the other antigenic groups.

No CF tests have been performed at this time; extensive studies done previously had shown no cross-reactions between viruses belonging to these 5 antigenic groups, except for a small crossing repeatedly observed between antisera for Ganjam virus and a strain of CCHF virus, C-3010.

The present observations show, particularly by HI, the existence of relationships between antigenic groups of viruses from the Bunyavirus-like set or from the unclassified category (Berge, 1975); only tick-borne viruses are thus far involved. In our estimate the described relationships do not at this time justify combining the viruses of the 5 related groups in a single group; rather, as was our conclusion from the relationships observed with antigenic groups of Bunyaviridae, and for similar reasons (Casals, Proceedings of the 7th International Congress of Tropical Medicine and Malaria, 1964), it would seem advisable to consider these new relationships as indicating the existence of a set of loosely and distantly related antigenic groups, an antigenic supergroup which for the sake of simplicity is designated the Nairovirus supergroup.

The published evidence that any of the viruses in the Nairovirus supergroup are Bunyaviridae is very slim and on the whole only morphological for the Bunyavirus-like; less is even known about the basic properties of the unclassified viruses. It is, therefore, premature and unjustified to assign ICTV taxonomic status--genus or family--to the new supergroup until biochemical and molecular studies determine the basic properties of the component viruses.

Table 1

Nairovirus supergroup antigenic relationships

Serum	Test and Antigen							
	HI					NT		
						CCHF		Bandia
	CCHF	Hazara	Dugbe	A.Minah	Other	Mouse	RFFI	Mouse
CCHF	320	80	20	40	0		+	0
Hazara	40	160	40	10	0			0
NSD						+	+	
Ganjam	80	20	40	0	0		±	
Dugbe	160	80	80+	20	0		+	0
A. Hammad	40+	20	80	40+	0			0
A. Minah	160	40	160					0
DGK	20	0	20	0	0			
Kao Shuan	40	5	20	10	0			
Pretoria	40	10	40	20	0			0
Pathum Thani	0	0	0	0	0	0	0	
Bandia	40	5	20	0	0	+	±	+
Qalyub	320	40	320	40+	0	+		
Farallon	10	5	20	0	0			0
Hughes	20	5	20	0	0	±		
Soldado	5	0	20	0	0	+	±	
Sakhalin	5	0	10	0	0			
Others, 34	0	0	0	0	+	0/4	0/5	

CCHF: neutralization test in mice, negative sera; Bhanja, CTF, Huacho and Uukuniemi.

CCHF: RFFI, reduction of fluorescent foci of infection, negative sera; chikungunya, Huacho, Mono Lake, Tyuleniy and West Nile.

HI, other antigens: Bhanja, Germiston, Ketapang, Lanjan, Manawa, Naples SFF, Oropouche, Quaranfil and Uukuniemi.

Reciprocal of serum titers; 0, negative at 1:5 or 1:10.

Table 2

Nairovirus supergroup antigenic relationships
Immunofluorescence test

Antigenic group	Virus	Antigen							
		CCHF	HAZ	DUG	A.HA	A.MIN	QAL	FAR	HUGH
CCHF	CCHF	256	8	4	0	0	0	0	0
	Hazara	32	64	0	0	0	?	0	0
NSD	NSD								
	Ganjam	4	2	16					
	Dugbe	16	2	128	0	0	?	4	0
DGK	Abu Hammad	0	0	0	256	64		und	0
	Abu Minah	0	0	0	64	256			
	DGK		2	0	32+	32			
	Kao Shuan	0	0	0	8	8			
	Pretoria Pathum Thani	0	0	0	32	64			
Qalyub	Bandia	0	0	0	0	0	+	0	?
	Qalyub	0	0	0	0	0	32+		
Hughes	Farallon	0	0	0	0	0	0	256	
	Hughes	0	0	0	0	0	0		256
	Soldado	0	0	0	0	0	0		
Sakhalin				0	0	0	0		
Others, and ungrouped		0	0	0	0	0	0	0	

Reciprocal of serum titer; 0, negative with undiluted serum.
?, questionably positive; +, positive at dilution 1:4,
but not titrated.

Serologic relationship between Rift Valley fever virus and viruses of the phlebotomus fever serogroup (R. Shope and C.J. Peters). A serologic relationship of Rift Valley fever has been found with viruses of the phlebotomus fever group in the Bunyaviridae. Rift Valley fever virus previously was believed on the basis of complement-fixation (CF) tests to be unrelated serologically to other bunyaviruses.

A Rift Valley fever (RVF) hemagglutinating antigen and antigens of phlebotomus fever group viruses were reacted with hyperimmune mouse ascitic fluids of phlebotomus fever group viruses (some of these reagents were supplied by the Research Resources Branch of the US NIH) and sheep anti-RVF serum. Inhibition of RVF antigen and Punta Toro, Chagres, and Saint-Floris and other antigens was observed when antibody of Punto Toro, Candiru, and Gordil (closely related to Saint-Floris virus) were used. A phlebotomus fever grouping antibody inhibited the RVF antigen in the 1:160 dilution. (Table 3).

Complement-fixation tests in grid titrations with Punta Toro and Gordil antigens and antibodies failed to detect cross-reactions with RVF reagents. Homologous antibody titers were RVF sheep serum 1:32, Gordil and Punta Toro antibody 1:512.

Plaque reduction neutralization tests in Vero cells done by Dr. C.J. Peters of Ft. Detrick (Table 4) resulted in neutralization of RVF virus by both Punta Toro and Gordil ascitic fluids as well as other antibody to phlebotomus fever group viruses. It has not yet been determined if any of the 26 distinct viruses in the phlebotomus fever group are identical to RVF, nor has the full range of relations within the group been explored.

Possible implications of these findings are 1) that heterologous reactions of phlebotomus fever group viruses may confound interpretation of RVF diagnostic reactions and serosurvey results, especially when HI and neutralization tests are involved, 2) that one or more viruses of the phlebotomus fever group might serve as a heterologous vaccine or might play a role in regulating the distribution of RVF infections because of cross-immunity of vertebrate hosts, 3) that RVF virus might have a natural reservoir in a phlebotomine fly, reasoning by analogy with the natural history of some phelbotomus fever group viruses which are maintained transovarially in phlebotomines.

Table 3

HI Test Reactions of RVF Virus and Phlebotomus Fever Group Viruses

	Antibody								
Antigens	RVF	SFS	SFN	CHG	ICO	PT	CDU	GOR	GP PHL
RVF	>640[a]	0	0	0	0	640	40	160	160
SFS	0	40	10	0	10	0	0	0	20
CHG	40	0	10	10	10	40	0	20	40
PT	40	0	20	0	0	640	20	0	80
SAF	320	0	40	0	0	40	40	160	80
SAL	20	0	0	0	0	40	10	0	80
KAR	0	0	0	0	0	40	0	0	80
I47	20	0	20	0	0	20	20	20	20

[a]Reciprocal of antibody titer; 0=<10; 2 - 4 antigen units. RVF antigen was produced at USAMRIID. Abbreviations: RVF, Rift Valley fever; SFS, Sicilian; SFN, Naples; CHG, Chagres; ICO, Icoaraci; PT, Punta Toro; CDU, Candiru; GOR, Gordil; GP PHL, group phlebotomus fever; SAF, Saint-Floris; SAL, Salehabad; KAR, Karimabad; I47, Naples-like isolate.

Table 4

Neutralization of RVF Virus by Antisera to Phlebotomus Fever Group Viruses[a]

	Antibody									
Virus	RVF	PT	GOR	CDU	ICO	SFN	SFS	SAL	GP PHL	NORMAL MOUSE
RVF	10, 240[b]	320	80	40	40	10	10	<10	640	<10

[a]69 to 104 pfu of RVF were incubated 1 hr at 37°C with the antibody before assaying residual infectivity in VERO cells to determine the highest dilution neutralizing 80% of the inoculum. Data from Dr. C. J. Peters, Ft. Detrick, Md.

[b]Reciprocal of titer; abbreviations as in Table 3.

II. Virus identification

FIJI-41451 (B.Q. Chen, S.M. Buckley and J. Casals). This agent was submitted for identification by Dr. R.B. Tesh, Pacific Research Unit, Honolulu, Hawaii; considered by him as a probable strain of Ross River virus. The virus was isolated, in the course of an epidemic of polyarthritis in Fiji, from the serum of a patient by inoculation to mosquitoes, then passed to VERO cells; the material sent to YARU was the VERO cell passage one.

The material was inoculated to several cell cultures: VERO, BHK-21, CER, A. albopictus (C6/36) and A. pseudoscutellaris (MO 61); replication occurred in all, but CPE was noted only with VERO and BHK-21. Spot-slides were prepared with all the inoculated cells and tested by immunofluorescence (IF) against dilution 1:4 of the following grouping polyvalent ascitic fluids: group A, group B, California group, phlebotomus group, Sakhalin group, VSV group and NIH polyvalent reagents 1, 2, 4, 5, 6 and 9. Only polyvalent grouping fluid A gave positive reaction, with all the spot-slides, clearly establishing the group affiliation of the strain.

Culture fluids from infected BHK-21 and VERO cells agglutinated goose erythrocytes at a pH range from 6.0 to 7.0 for BHK-21 and from 6.0 to 6.6 for VERO; at the optimal pH, 6.4, the titer of the HA antigen was 1:64.

A hyperimmune serum for Fiji-41451 is being prepared in mice by inoculation of tissue culture propagated virus. Attempts to propagate the virus in mice are also under way; after 3 consecutive intracerebral passages a long incubation--10 days--transmissible agent has been established the nature of which is to be determined.

CS 122 (A.Brescia and J. Casals). This agent was submitted by Dr. R.L. Doherty, University of Queensland, Australia. The virus had been isolated by Dr. T. St. George, CSIRO, from Ixodes uriae nymphs collected at Macquarie Island on December 1976, identified as a flavivirus and distinguishable from Australian agents.

An HA antigen has been prepared from infected newborn mouse brain tissue with a pH range from 6.8 to 7.0 or higher; the titer of this antigen at pH 7.0 is 1:40. By CF the titer of this antigen is 1:16.

Confirmation of the antigenic group affiliation of CS 122 was easily and rapidly accomplished by immunofluorescence, by testing an antiserum for the strain in dilutions beginning at 1:4 against polyvalent groups A and B slides; the serum reacted with the group B slide with a titer of 1:16 while it was negative against group A.

Further serological characterization of this strain is continuing. Hyperimmune sera or ascitic fluids for several flaviviruses have been tested against CS 122 antigen by CF; the result of a test is shown in Table 5. The low titer of two immune reagents, SLE and Omsk hemorrhagic fever, preclude any conclusions regarding the relationship of these two viruses with CS 122; otherwise, it is clear that CS 122 differs markedly from dengue 2 and Tyuleniy and is close to a strain of Central European tick-borne encephalitis. It is planned to test by CF as soon as possible an antiserum for CS 122 against all flavivirus available in this laboratory.

Strains MVE-Aa and Kunjin-Aa. (J. Casals). Drs. A.J. Gibbs and M.W. Davey, The Australia National University, Canberra, Australia, requested this laboratory's assistance to confirm the identity of two strains, here designated MVE-Aa and Kunjin-Aa respectively, which had been maintained in their laboratory in chronically infected A. albopictus cells by weekly passage for over one year.

Materials from Australia were received in this laboratory as cell cultures MVE-Aa passage 50 and Kunjin-Aa passage 50. Fluids and cells from each flask were harvested together; newborn mice were intracerebrally inoculated with each culture and the rest of the fluids with debris were saved to use as antigens in a CF test.

The mice inoculated with either culture first appeared sick on the 4th day after inoculation, became worse and began to die on days 5, 6 and 7. Crude frozen-thawed CF antigens were prepared with both viruses by making a 10% suspension in physiological saline, freezing and thawing 3 times for periods of 10 minutes and centrifuging at 5000 rpm for 30 minutes; the supernatant fluids were used as antigens.

The fluids and debris from the original tissue cultures were used as antigens in a CF test; reference immune reagents for Kunjin and MVE viruses were used, with homologous titers of 1:32 and 1:16, respectively. The results of the test were negative, indicating that no CF antigen was detectable in the Kunjin-Aa and MVE-Aa fluids.

A second CF test was carried out with the following reagents:

Antibodies: MVE, mouse immune serum, reference reagent, Jan. 1963.
Kunjin, mouse ascitic fluid, reference reagent, May 1966.

Antigens: MVE-Aa, mouse brain tissue, frozen-thawed
Kunjin-Aa, mouse brain tissue, frozen-thawed
MVE, sucrose-acetone, reference antigen
Kunjin, sucrose-acetone, reference antigen
MVE, 10% stock virus suspension, July 1965
Kunjin, 10% stock virus suspension, June 1962
As control, Zirqa sucrose-acetone antigen

The results of the CF test is given in Table 6. From this test it is evident that both MVE-Aa and Kunjin-Aa behaved as Kunjin; MVE-Aa did not match YARU's reference reagents for MVE virus.

Table 5

Complement-fixation test
Relationships of CS 122 strains with other group B viruses

Antigen	Immune Serum					
	CS 122	D2	SLE	CETBE	OHF	Tyuleniy
CS 122	32/16	0	4/2	32+/8	4/2	4/2
Homologous	32/16	32+/16+	32+/16+	32+/16+	4/16+	32+/16+

Reciprocal of serum titer/reciprocal of antigen titer.
First dilution of CS 122 antigen 1:2; first dilution of immune sera 1:4.

Table 6

Complement-fixation test
Identification of strains Kunjin-Aa and MVE-Aa

Antigen	Serum	
	MVE	Kunjin
MVE-Aa, frozen-thawed	8/2	32/32
Kunjin-Aa, frozen-thawed	32/16	32/32
MVE, SA reference	256/64+	16/64+
Kunjin, SA reference	64/16	64/64+
MVE, virus stock	256/16+	8/8
Kunjin, virus stock	8/2	32/16+
Zirqa, Sa, control	0	0

Reciprocal of serum titer/reciprocal of antigen titer

Teh 193-4, Teh 193-116 and Teh 193-172 (P. Sureau and J. Casals). These strains were isolated in Iran, province of Khorassan, in 1978 by Dr. P. Sureau, then at the Institute Pasteur, Teheran. The initial phases of the characterization of the strains was done in Teheran; final characterization at this laboratory.

Teh 193-4 was one of two serologically identical strains, both isolated from Hyalomma asiaticum males; by CF test the strain crossed to titer with the Wanowrie reference strain. Teh 193-116 was one of 4 similar strains isolated from Argas vulgaris; the strain had been provisionally identified as Quaranfil and this was confirmed at YARU by CF test: the strain cross-reacted to titer with the Quaranfil reference virus. Teh 193-172, isolated from H. anatolicum females, was identified by CF as a strain of Wad Medani.

Mazoe virus: serological relationship with IPPY virus (J. Casals). Mazoe virus was isolated in Rhodesia from wild-caught murids (Swanepoel et al., 1978, J. Hyg., Cam, 80: 183); the virus had remained antigenically ungrouped and taxonomically unclassified. IPPY virus, also ungrouped and unclassified was isolated in 1970 in Central African Republic from blood and viscera from an Arvicanthus (Digoutte and Pajot, Working Arbovirus Catalogue, May 1975). These two viruses share a property, a 10-12 day incubation period following intracerebral inoculation to newborn mice; this fact and certain ecological considerations brought to our attention by Dr. Swanepoel stimulated a serological comparison by CF test between the two agents. The initial results of this as yet incomplete investigation is shown in Table 7. The Mazoe antigen was a sucrose-acetone preparation, those for IPPY crude 10% suspensions of infected mouse brain centrifuged at low speed; as the table shows, there was a one way crossing in this test with the IPPY serum reacting with the Mazoe antigen. It is somewhat disturbing that the titer of Mazoe antigen was 1/4 with its homologous serum and much higher, 1/32, with the IPPY serum. Additional work on this problem is under way.

Tettnang (KOLN 63) virus: relationship with mouse hepatitis (J. Casals). A virus strain, KOLN 63, submitted to this laboratory for identification in 1972, by Drs. B. Rehse-Kupper and R. Ackermann, Cologne, Germany, was at the time shown to be serologically unrelated to all viruses with which it was compared in this laboratory, except a strain sent by Dr. R. Williams, NAMRU-3, Cairo, Eg Art 1147; these two strains were indistinguishable by CF test. Neither of these strains had been screened here at the time for the possible presence of mouse hepatitis virus.

Investigations by Dr. V. Bardos, Institute of Parasitology, Prague, which he communicated to us in 1979, appeared to indicate a serological relationship between Tettnang, KOLN 63, and mouse hepatitis viruses. This has been confirmed in this laboratory, Table 8 , in so far as it concerns the KOLN 63 strain; no tests have been done with the NAMRU-3 strain. It is not known whether the original

Table 7

Complement-fixation test
Relationship between Mazoe and IPPY viruses

Antigen	Serum	
	Mazoe	IPPY
Mazoe, sucrose-acetone	64/4	8/32
IPPY, crude, sample 1	0	128+/16+
IPPY, crude, sample 2	0	128+/16+
Diluent	0	0

Reciprocal of serum titer/reciprocal of antigen titer
0, no reaction with dilution of serum 1:4 and undiluted antigen.

Table 8

Complement-fixation test

Cross-reaction between Tettnang and mouse hepatitis viruses

Antigen	Serum		
	KOLN 63	M.hepat.#1	M.hepat.#2
Tettnang, KOLN 63	16/32	32/64	4/16
Mouse hepatitis, BT 25	8/8	32/16	4/8
Mouse hepatitis, #2	4/32	16/64	16/64
Mouse brain, control	0	0	0
No antigen	0	0	0

Reciprocal of serum titer/reciprocal of antigen titer

isolate from Germany was, or was contaminated with, mouse hepatitis or whether the contamination occurred in this laboratory; in fact it is not possible to state whether there ever was a Tettnang virus until additional studies are done.

KT 281/75 (J. Casals). This strain, submitted by Dr. D.I.H. Simpson, The London School of Hygiene and Tropical Medicine, London, had been isolated from Amblyomma variegatum ticks in 1975 in Kenya. An antigen for this agent was screened by CF test against 19 group polyvalent sera or ascitic fluids as well as against a CCHF (IbAr 10200) hyperimmune sera. All reactions were negative except with the CCHF antiserum which reacted with a titer of 1:8 against the KT 281/75 antigen.

Additional CF tests showed that an immune ascitic fluid for KT 281/75 reacted weakly with Congo-Crimean hemorrhagic fever and Hazara antigens, but strongly with Dugbe antigen; and a hyperimmune serum for Ganjam (Nairobi sheep disease) virus gave a negative reaction with a KT 281/75 antigen. A final CF test, Table 9, clearly showed that this strain and Dugbe (IbAr 1792) virus are indistinguishable by this method; KT 281/75 is tentatively considered a strain of Dugbe.

Brest Ar/T 101 (K. Obom and J. Casals). Strain isolated from O.A. maritimus ticks collected in Brittany (France), 1978, submitted by Dr. C. Chastel with the information that the strain although closely related to Soldado Rock virus by CF, appeared to be distinguishable from it; Dr. Chastel requested re-examination and confirmation.

Screening by CF in this laboratory with reference polyvalent ascitic fluids promptly showed that this strain belonged in the Hughes group. Furthermore, an antigen for Brest Ar/T 101 was tested against antisera from Hughes and Soldado Rock viruses; a marked cross-reaction occurred with the Soldado Rock antiserum, hardly any with the Hughes. Additional work is in progress to complete the characterization of the strain.

BeAn 327600 virus (E. Kling and R. Shope). This virus was submitted by Dr. Francisco Pinheiro of Belem, Brazil. It was isolated from a rodent and identified as a group B agent. At YARU a mouse brain antigen was produced with optimum HA at pH 6.8. In an HI test the group B relationship was confirmed and inhibition was noted with ascitic fluids to SLE (1:320), Ilheus (1:320) and Bussuquara (1:20); Powassan was negative. Results of a CF test are shown in Table 10. By CF BeAn 327600 is related to but different from SLE and Ilheus. It is not dengue 2, dengue 3 or Jutiapa. In a neutralization test in baby mice BeAn 327600 ascitic fluid with a homologous LNI of 4.1 did not neutralize Ilheus virus. BeAn 327600 is probably a new group B virus although further tests are needed to confirm this.

Table 9

Complement-fixation test
Identification of strain KT 281/75

Antigen	Serum	
	KT 281/75	Dugbe
KT 281/75	128/256	256/256
Dugbe	128/128	512/64
Control	0	0

Reciprocal of serum titer/reciprocal of antigen titer.

Table 10

CF reactions of BeAn 327600 virus

	Ascitic fluids					
Antigens	BeAn 327600	Ilheus	St. Louis	Den-2	Den-3	Jutiapa
BeAn327600	256[a]	32	64	0	0	4
Ilheus	32	256	8			
St. Louis	32	32	256			
Den-2				64		
Den-3					16	

[a]Reciprocal of ascitic fluid titer.

Identification of viruses from Australia (D. Cybinski, J. Wright, C. Reed, and R. Shope). Six viruses from Australia were tested by CF with grouping and polyvalent mouse ascitic fluids. The viruses were:

CSIRO 25	from Culicoides peregrinus
CSIRO 79	from Lasiohelea spp.
CSIRO 132	from Culicoides brevitarsis
Ch 16287	from Culex annulirostris
Ch 19520	from Culex annulirostris
Ch 19546	from Culex annulirostris

Ch 19546 antigen reacted with polyvalent Anopheles A, Anopheles B, Turlock grouping fluid. This result confirmed the observation at the Queensland Institute for Medical Research that Ch 19546 was in the Turlock group.

The other 5 antigens reacted positively with their homologous ascitic fluids and were negative at 1:4 with fluids of group A, group B, group phlebotomus fever, group Simbu, group VSV (Indiana, New Jersey, Cocal), group Tacaribe, (Tacaribe, Junin, Amapari, Pichinde, Tamiami), group Sakhalin, group Bunyamwera, group Kemerovo, polyvalent Quaranfil (Quaranfil, Kaisodi, Bandia, Johnston Atoll, Qalyub, Silverwater, Lanjan), polyvalent Anopheles A (Anopheles A, Lukuni, Anopheles B, Boracea, Tacaiuma, CoAr 1071, CoAr 3624, Turlock, Umbre, M'Poko), Polyvalent Bwamba (Bwamba, Pongola, Mossuril, Kamese, Eretmapodites 147, Nyando), polyvalent Palyam (Palyam, Vellore, Kasba, D'Aguilar, Corriparta, Pata, Eubenangee, Acado), polyvalent rabies, LCM, herpes, NDV, vaccinia, polyvalent Patois, Zegla, Shark River, Mirim, Bertioga, polyvalent Congo, Hazara, Ganjam, Dugbe, Bhanja, and polyvalent fluids with antibody to (1) Bahig, Tete, Matruh, Matariya, Burg el Arab, (2) Jurona, Minatitlan, Gamboa, Juan Diaz, Belem, (3) Koongol, Wongal, Bakau, Ketapang, Mapputta, Trubanaman, Maprik, (4) Nyamanini, Uukuniemi, Grand Arbaud, Thogoto, (5) Hughes, Sawgrass, Matucare, Lone Star, Soldado, (6) Marco, Timbo, Chaco, Pacui, (7) Hart Park, Flanders, Kern Canyon, Klamath, Mt. Elgon bat, (8) bluetongue, EHD, IbAr 22619, Changuinola, Irituia, Colorado tick fever, (9) Navarro, Triniti, Aruac, Pacora, (10) Upolu, DGK, Wanowrie, Dhori, (12) Okola, Olifantsvlei, Witwatersrand, Bobia, Tataguine, Polyvalent Joinjakaka, Mitchell River, Warrego, Japanaut, Belmont, Wallal, Wongorr, Charleville, Almpiwar and to mouse hepatitis.

CSIRO 132 antigen was also negative by CF with antibody to kotonkan (homologous >128), Chandipura (16), Barur (>128), Kwatta (>128), Lagos Bat (>128), Piry (>128), Obodhiang (>128), Kimberley (64), Porton S-1643 (16), Keuraliba (128), and BeAr 185559. CSIRO 132 had been shown previously by CSIRO to be a rhabdovirus. Attempts at Yale to visualize the virus by EM in mouse brain were not successful.

Attempts to produce hemagglutinating antigens by sucrose-acetone extraction of mouse brain with the 6 Australian viruses were negative using goose cells and pH range of 5.75 to 7.2 at .15 M and .4 M.

Particles with Bunyavirus-like morphology and morphogenesis were visualized by thin-section electron microscopy of Ch 19520 virus.

GaAr 79V1463 virus (L. Coimbra and R. Shope) submitted by Dr. Charles Calisher of CDC, Ft. Collins was isolated from mosquitoes in the Gambia. This virus reacted by CF with the grouping ascitic fluid containing antibody to Tataguine virus and was subsequently typed as Tataguine virus at CDC.

Philippines Ar 281 and Ar 814 viruses (F. Danielle, C. Bevin, and R. Shope). These viruses were submitted by Dr. James Olson of NAMRU-II, isolated from mosquitoes in the Philippines. Provisional identification at NAMRU-II indicated that Ar 281 was Japanese encephalitis virus and that Ar 814 was Getah virus. CF tests at YARU confirmed that Ar 281 was a group B virus reactive equally with JE and West Nile viruses and different from Zika virus. A neutralization test in mice gave the following result:

Ascitic fluids	Virus			
	JE		Ar 281	
	titer in log LD_{50}	LNI	titer in log LD_{50}	LNI
Normal	7.4	-	5.3	-
JE (Nakayama)	5.2	2.2	3.4	1.9
Ar 281	3.7	3.7	1.8	3.5

The results indicate that Ar 281 virus is identical to Japanese encephalitis virus. This is the first time to our knowledge that JE virus has been isolated in the Philippines.

CF tests with Ar 814 indicate a close relationship and probably identity with Getah virus however a low titer of Ar 814 brain CF antigen has precluded final identification by this test.

Central African Republic ANCB-672d virus (P. Sureau, G. Tignor, and A. Smith). This isolate from a Micropterus pusillus bat was provisionally identified as Lagos bat virus at the Institut Pasteur, Dakar. Since the only other existing strain was isolated 18 years before from a different species of bat (Eidolon helvum) in Nigeria, the new isolate was compared by kinetic neutralization to search for strain differences. Two inoculation mouse sera and the fluorescent focus inhibition test in CER cells were used to compare the 2 Lagos bat strains and rabies, Mokola, and Duvenhage viruses. The least significant difference between mean titers and the regression analysis by the least squares method allowed comparison of slopes of

the regression lines, indicating the affinity of each antibody for each virus.

The affinity of Lagos bat prototype serum for the homologous virus and for ANCB-672d was indistinguishable (Table 11); however the affinity of ANCB-672d serum is greater for the homologous than for the prototype Lagos bat virus (Table 12).

An additional difference was observed when the 2 sera were tested with Mokola virus; ANCB 672d serum reacted more rapidly than the prototype Lagos bat serum (Table 13); the reciprocal was also true. Sera of Duvenhage and the CVS strain of rabies virus reacted equally with the 2 Lagos bat virus strains (Table 14).

In addition, reciprocal vaccination-challenge tests in mice did not distinguish the 2 Lagos bat strains.

Identification of viruses from Connecticut including Connecticut virus of the Sawgrass Group (A.J. Main). Ten strains of virus were recovered from mosquitoes collected in Connecticut during 1979. Flanders virus was isolated from a pool of Culiseta melanura from Farmington; the remaining nine isolates were from mosquitoes trapped along the Hammonasset River in North Madison. These included Flanders virus from three pools of Cs. melanura and one pool of Culex restuans; Highlands J virus from a pool of Cs. melanura; Jamestown Canyon virus from two pools each of Aedes abserratus and Ae. aurifer; and an untyped Bunyamwera group virus from Ae. triseriatus. This is the first report of a Bunyamwera group virus in New England.

Two strains of virus were recovered from ticks collected during 1978--both from the same site in Old Lyme. The first was an isolate of Powassan virus from a pool of 9 Ixodes cookei numphs from a long-tailed weasel (Mustela frenata) trapped on 7 October 1978. Powassan virus was recovered from the blood of the weasel. However, the titer of virus was higher in the tick than in the blood suggesting that the tick was infected. Virus was not isolated from a pool of 23 larval I. cookei or 4 larval I. dammini removed from this weasel at the same time as the nymphs.

A Sawgrass group virus was isolated from a pool of 21 I. dentatus nymphs collected from two nonviremic eastern cottontails (Sylvilagus floridanus) trapped on 22 August 1978. This isolate (Ar 1152-78) was serologically distinct from both Sawgrass and New Minto viruses by CF and neutralization (Table 15) tests and has been named Connecticut virus.

Table 11

Kinetic NT with Lagos bat (prototype) serum

Virus	Regression Analysis			
	Coef icient of determination	Slope		
		Slope	SE	95% confidence range
LB (prototype)	1.0	-0.1286	0.0083	-0.1369 to -0.1203
LB (ANCB-672d)	0.99	-0.1206	0.0185	-0.1391 to -0.1021
MOK (IbAn 27377)	0.63	-0.0150	0.0224	-0.0374 to +0.0074
Rabies (CVS)	0.74	-0.0106	0.0186	-0.0292 to +0.0080
DUV	0.95	-0.0152	0.0060	-0.0212 to -0.0092

Table 12

Kinetic NT with Lagos bat (ANCB-672d) Serum

Virus	Regression Analysis			
	Coefficient of determination	Slope		
		Slope	SE	95% confidence range
LB (prototype)	0.98	-0.0816	0.0127	-0.0943 to -0.0689
LB (ANCB-672dO	1.0	-0.1152	0.0070	-0.1222 to -0.1082
MOK (IbAn 27377)	0.99	-0.0844	0.0176	-0.1020 to -0.0668
Rabies (CVS)	0.88	-0.0328	0.0238	-0.0566 to -0.0090
DUV	0.84	-0.0270	0.0219	-0.0489 to -0.0051

Table 13

Kinetic NT with Mokola (IbAn 27277) Serum

	Coefficient of determination	Regression Analysis		
		Slope		
		Slope	SE	95% conficence range
LB (prototype)	0.79	-0.0222	0.0223	-0.0445 to -0.0001
LB (ANCB-672d)	0.98	-0.0830	0.0245	-0.1075 to -0.0585
MOK (IbAn 27377)	1.00	-0.1746	0.0172	-0.1918 to -0.1574
Rabies (CVS)	0.91	-0.0168	0.0103	-0.0271 to -0.0065
DUV	0.93	-0.0108	0.0119	-0.0227 to +0.0011

Table 14

Summary of kinetic NT with rabies-related viruses

Serum	Virus	*$LN \frac{Vt_{20}}{Vo}$	Regression Analysis		
			slope	Coefficient of determination	intercept
LB (prototype)	LB (prototype)	-2.56	-0.13	1.0	-0.06
	LB (ANCB-672d)	-2.44	-0.12	0.99	-0.13
	MOK (IbAn 27377)	-0.36	-0.02	0.63	+0.05
	Rabies (CVS)	-0.25	-0.02	0.74	+0.08
	DUV	-0.28	-0.02	0.95	+0.03
LB (ANCB-672d)	LB (prototype)	-1.62	-0.08	0.98	-0.03
	LB (ANCB-672d)	-2.24	-0.12	1.0	+0.07
	MOK (IbAn 27377)	-1.76	-0.08	0.99	+0.01
	Rabies (CVS)	-0.59	-0.03	0.88	+0.12
	DUV	-0.63	-0.03	0.84	+0.02
MOK	LB (prototype)	-0.45	-0.02	0.79	-0.08
	LB (ANCB-672d)	-1.79	-0.08	0.98	-0.06
	MOK (IbAn 27377)	-3.47	-0.17	1.0	-0.06
	Rabies (CVS)	-0.32	-0.02	0.91	+0.05
	DUV	-0.21	-0.01	0.93	+0.03
Rabies (CVS)	LB (prototype)	-0.44	-0.004	0.77	-0.02
	LB (ANCB-672d)	-0.44	-0.02	0.80	-0.08
	MOK (IbAn 27377)	-0.32	-0.02	0.91	+0.05
	Rabies (CVS)	-3.71	-0.19	1.0	0.08
	DUV	-0.41	-0.02	0.94	0.04
DUV	LB (prototype)	-0.02	-0.004	0.05	-0.02
	LB (ANCB-672d)	-0.85	-0.04	0.94	+0.02
	MOK (IbAn 27377)	-0.16	-0.01	0.89	+0.01
	Rabies (CVS)	-1.42	-0.07	0.99	+0.08
	DUV	-1.04	-0.05	0.96	-0.03
NMS	LB (prototype)	-0.20	-0 002	0.97	+0.02
	LB (ANCB-672d)	-0.23	-0 01	1.0	+0.01
	MOK (IbAn 27377)	-0.08	-0 004	0.57	+0.03
	Rabies (CVS)	-0.01	-0 01	0.57	-0.04
	DUV	-0.10	-0 004	0.04	-0.10

*Natural log of the surviving virus at time 20 minutes.

Table 15

Serological identification of USA Ar-1152-78 from Ixodes dentatus ticks collected in Connecticut during 1978

Complement-fixation tests:

	ASCITIC FLUIDS		
	Ar 1152-78	Sawgrass	New Minto
Ar 1152-78	256/128*	32/64	0
Sawgrass	16/32	128/32	32/32
New Minto	4/16	0	256/512

* Reciprocal of serum titer/reciprocal of antigen titer.

Neutralization Test in Suckling Mice:

		ASCITIC FLUIDS		
		Ar 1152-78	Sawgrass	New Minto
Ar 1152-78	(5.1)*	≥3.5**	1.1	0.9
Sawgrass	(4.3)*	2.5	≥2.8	1.3
New Minto	(5.2)*	1.9	1.8	≥3.7

* Log of LD_{50} in suckling mice (ic)

** Log of the neutralizing index

Identification of viruses in field material from Ethiopia (O. Wood). The Naval Medical Research Unit #5 submitted for identification, viruses isolated from arthropods and animals as part of a broad ecologic study of disease in Ethiopia. After closure of NAMRU-5, materials remaining in the freezers were shipped to Yale where mosquitoes were pooled and inoculated into mice for virus isolation attempts. In addition, isolates from these pools and from materials already processed in Ethiopia were to be identified. To date, most of this material has been processed and many of the isolates identified (see previous reports).

Five viruses represented by prototype EthAr 792 kill baby mice but do not cause cytopathic effect and/or antigen (as detected by FA) in Vero, Aedes albopictus, Aedes pseudoscutellaris, C6, N18 neuroblastoma, or BHK-21 cells. CF tests with 20 arbovirus grouping ascitic fluids representative of African viruses were negative. These viruses were not inactivated by desoxycholate in tests done in Ethiopia and remain unidentified to date.

At least 2 additional agents were isolated at YARU from Ethiopian mosquito pools. These agents have not been filtered to prove their viral nature, nor have they been identified serologically.

Three viruses, EthAn 3024 and EthAn 3490 from rodents and EthAr 4848 from mosquitoes are negative in CF tests with grouping fluids. EthAn 3490 and EthAr 4848 are identical by CF tests.

Another ungrouped virus, EthAn 3554 is not sensitive to desoxycholate and will be tested for acid sensitivity.

EthAr 5348 from mosquitoes reacted by CF with Bwamba antibody. A neutralization test with Bwamba and Pongola virus is scheduled to type the agent.

III. Serological surveys

Arbovirus antibody survey in China (B.Q. Chen and J. Casals). Sera from 91 persons were collected in 1979; 76 were from residents of Peking or environs and derived from healthy persons or from patients in infectious diseases or children's wards, 8 were from individuals in Inner Mongolia and 7 from Sinkiang and Tibet. The numbers are not suffiently large to justify a break down in terms of place of origin, age, sex or health status; the group must be considered as a whole, as an indicator of probable arbovirus activity in China.

The sera were tested by hemagglutination-inhibition (HI) against 8 units of each of the following antigens: group A (alphavirus), chikungunya, EEE, Ross River and Sindbis; group B (flavivirus), dengue 2, JE, KFD, MVE, Omsk hemorrhagic fever, Russian spring-summer encephalitis, Sepik, West Nile and yellow fever; Bunyamwera group, Batai; California group, California encephalitis; and phlebotomus fever group, Naples sandfly fever virus.

The sera were screened at dilution 1:10 against all the antigens; those found positive were titrated in increasing two-fold dilutions.

To interpret the HI results with the flavivirus antigens the following criteria were followed. A serum was considered to have given a diagnostic reaction for one of the antigens included in the tests when the titer against this antigen was at least 2 dilutions (4-fold) higher than against any of the other antigens of the group. A serum that reacted with several antigens with similar titers was considered to indicate a superinfection, i.e., exposure in succession to two or more viruses of group B, if the titers were relatively high, for example, 1:80 or higher. A serum that gave a titer of 1:10 or 1:20 against one or several antigens was considered to indicate exposure to an undetermined virus of the group. A serum that reacted equally well with JE and MVE and was negative or gave very low titer with other antigens could not be in fact diagnosed, except that in the present context we were inclined to consider the antibodies due to exposure to JE virus rather than to MVE or to both agents. A similar reasoning was applied to sera with antibodies against JE and WN viruses.

Sera were positive only against groups A and B viruses; the results are summarized in Table 16 and representative examples are shown in Table 17.

One serum was positive against chkungunya and another against EEE viruses (the latter also had antibodies for flaviviruses); the epidemiological significance of these isolated reactions cannot be assessed. No positives were detected against Ross River or Sindbis viruses.

Table 16

Hemagglutination-inhibition test
Human sera from China
Survey for antibodies against selected arboviruses

Number of sera	91		
Negative for all antigens		45	
Positive for an antigen		46	
Positive group A			2
Chikungunya			1
EEE			1
Positive group B			45
JE			15
JE-MVE			3
JE-MVE-WN			10
Superinfection			2
Undiagnosable			15

A serum was positive for groups A and B; hence the total for those 2 groups is 47 while the total for an antigen is 46.

Table 17

Hemagglutination-inhibition test
Human sera from China
Representative results

Serum		Antigen										
No.	Diagnosis	JE	MVE	WN	RSSE	YF	D2	KFD	OHF	SEP	CHIK	EEE
3	JE	40										
34	JE	320	80	80	10	20	10			10		
42	JE	320	40	20								
90	JE	80		10								
21	JE-MVE	80	80	10								
69	JE-MVE	40	40	10								
25	JE-MVE-WN	80	160	40								
85	JE-MVE-WN	40	10	40								
108	JE-MVE-WN	40	10	20								
22	Superinf.[a]	320	320	320	160	320	320	80	160	320		
26	Superinf.	80	80	40	10	20	10	10		20		
16	Undiag.[b]	10										
18	Undiag.	10	10	10								
19	Undiag.	20	10	10								
31	Undiag.		20		10							
65	Undiag.		10	10								
23	EEE-GB	40	20	10								40
45	CHIK										160	

Reciprocal of serum titer
Blank spaces: negative at 1:10, lowest dilution used.

[a]Superinfection

[b]Undiagnosable

Of the 45 sera that reacted with group B antigens, 15 could be considered a result of exposure to JE virus under the criteria adopted and an additional 2 either to JE or MVE. Of the 2 sera that gave a superinfection reaction pattern, at least one (#22 in Table17) must be derived from a person who had undergone exposure to two different group B viruses.

The reported results showing group B antibodies in 45 of 91 sera tested indicate considerable activity of these viruses among the Chinese population; and also that the most likely candidate as producer of these antibodies is Japanese encephalitis virus, or a closely allied agent not included in the survey.

Arbovirus antibody survey in Liberia (J.D. Frame and J. Casals). A total of 99 sera were collected from professional, nursing and auxiliary staffs in a hospital in Monrovia; about one third of the donors were short term residents, the rest Liberian nationals. The sera in increasing two-fold dilutions beginning at 1:10 and extending to 1:80 were tested by hemagglutination-inhibition (HI) against 8 units of the following antigens: group A (alphavirus), chikungunya and Sindbis; group B (flavivirus), dengue 2, Wesselsbron, West Nile, yellow fever and Zika; Bunyamwera group, Bunyamwera and Germiston.

The diagnostic criteria for the interpretation of results were the same as above (Arbovirus antibody survey in China), except that categories of probables were added indicating sera that reacted at dilution 1:10 only with an antigen of a group. Since few antigens from each group were used, it is to be stressed that the diagnostic conclusions reached do not preclude other conclusions had additional antigens been used.

A considerable number of sera had antibodies against viruses of more than one antigenic group, usually against chikungunya and yellow fever; as a consequence the total number of diagnosed exposures, 92, is greater than the total number of individuals with any kind of arbovirus antibodies, 67.

A summary of the results is given in Table18, and representative examples of reactions in Table19.

The high number of positive, and probably positive, sera against yellow fever may in great part be due to vaccination; it is planned to look into the records from these persons in order to clarify the situation. There is no doubt, on the other hand, that infection with chikungunya--or with a closely allied virus as might be o'nyong-nyong--was a frequent occurrence among the group surveyed.

Survey for antibodies against Lassa fever virus in equatorial Africa (J.D. Frame and J. Casals). This is part of a continuing survey being conducted in Africa; sera from a country, Uganda, not previously surveyed have been tested this year. Between January 1,

Table 18

Hemagglutination-inhibition test
Human sera from Liberia
Survey for antibodies against selected arboviruses

Number of sera	99		
Negative for all antigens		32	
Positive for an antigen		67	
Positive group A			29
chikungunya			28
Sindbis			1
Positive group B			54
Yellow fever			26
Probable yellow fever			16
Wesselsbron			4
West Nile			1
Zika			3
Superinfection			4
Positive Bunyamwera group			9
Probable Germiston			7
Probable Bunyamwera			2

Many sera were positive for antigens from different groups.

Table 19

Hemagglutination-inhibition test
Human sera from Liberia
Representative results

Serum No.	Diagnosis	Antigens CHIK	SIND	D2	YF	WESS	WN	ZIKA	BUNY	GERM
2	Group B, superinfection			10	80+	40	40	10		
13	Group B, superinfection			40	80	320	160	80		
23	Yellow fever				40					
24	Yellow fever				40					
130	Yellow fever				80+					
1	YF, probable				10					
25	YF, probable: CHIK	80+			10					
22	YF, probable: GERM, probable				10					10
238	Wesselsbron					40				
52	Wesselsbron: CHIK	40			10	40				
41	Zika							40		
27	chikungunya	80+								
34	chikungunya	80+	10							
50	chikungunya: YF, probable	80+			10					
22	Sindbis		20							

Reciprocal of serum titer
Blank spaces: negative at 1:10, lowest dilution used.

and December 15, 1979 42 sera from Uganda and 35 from Liberia were tested by the immunofluorescence (IF) technique against a Lassa fever inactivated antigen supplied by CDC, Atlanta, Georgia; 6 sera were positive and 1 questionable, all from Liberia.

IV. Diagnosis of current arenavirus and arbovirus disease.

Pichinde virus (D. Bishop and J. Casals). Serial sera from a laboratory worker at the University of Alabama who developed an illness presumably associated with his work, were submitted to this laboratory for serological diagnosis. The patient had been working with Pichinde and other viruses and developed an acute, moderately severe febrile illness with onset on 4 October, 1979.

Samples of serum taken on 20 September, 1978, 20 March, 1979, 8 October, 1979, and 18 October, 1979, were tested by complement-fixation test against antigens for Pichinde, LaCrosse, yellow fever and a normal antigen; all sera were tested in increasing two-fold dilutions beginning at 1:4. The sera from 8 and 18 October, 1979, were positive with a titer of 1:32 atainst Pichinde antigen only; the remaining reactions were all negative at dilution 1:4, lowest used. This is the first evidence that Pichinde virus can cause disease in man; that it can cause inapparent infection had already been established in this laboratory (1978, Annual Report, YARU) and in another one.

An attempt was made to isolate virus from the acute serum sample (October 4, 1979) by intracerebral inoculation to newborn mice; no virus was isolated.

Diagnosis of LaCrosse encephalitis by immunofluorescence (B. Beaty, C. Gundersen, and J. Casals). A project on rapid diagnosis of LAC encephalitis is currently being developed in these laboratories under NIH grant AI 15641; results are reported here since they pertain also to the reference function. As part of the project, investigations have been initiated to ascertain the possible application of the indirect immunofluorescence (IF) test to the diagnosis of a current infection by LAC encephalitis virus.

As antigen were used slides prepared with BHK-21 cells infected with LAC virus by depositing approximately 3×10^4 cells on each of the circular areas of a 12-circle Cel-line slide (spot-slides); the slides were dried at 37C, fixed with acetone and stored at -60C.

Single, convalescent sera from 4 patients (see Table 20) and paired sera from 8 additional patients were tested in increasing two-fold dilutions beginning at 1:4. Some of the serum donors suspected at first, on clinical grounds, to have LAC encephalitis were subsequently not confirmed by laboratory tests. The results of the IF test shown in Table 20 agreed closely with those of other tests, HI and counter-immunoelectrophoresis, done elsewhere; at this writing it has not as yet been possible to bring together all the information available on these patients. The reading of the IF test was extremely clear and since, given the slides, the test can be executed and read in from 2 to 3 hours after reception of the sera, its future use as an aid to a diagnosis of LaCrosse encephalitis appears to be warranted.

Table 20

Serological diagnosis of LaCrosse encephalitis by the immunofluorescence test

Serum		Titer	Serum		Titer
K.	convalescent	1:64+	E.H.	9-23-79	0
D.	convalescent	1:64		10-24-79	1:512
L.	convalescent	1:64+	A.A.	8-12-79	0
R.	convalescent	1:64		9-13-79	$\frac{+}{0}$
B.B.	8-7-79	1:8+	J.A.	9-25-79	
	8-29-79	1:8+		10-1-79	1:64
B.C.	9-26-79	1:8+	L.B.	7-4-79	1:8
	10-8-79	1:8		8-3-79	1:8
H.B.	8-10-79	0	C.B.	9-20-79	0
	8-17-79	1:256		10-11-79	1:8
M.F.	7-6-79	1:128	K.Br.	7-7-79	0
	8-2-79	1:512		8-13-79	0
J.H.	9-10-79	1:8	K.Bu.	9-19-79	0
	9-21-79	0		10-20-79	0

0, negative reaction at dilution 1:4, lowest used.

Ross River virus infection in Fiji (H. Artrob and J. Casals). Seven sera from 4 Canadian tourists who visited Fiji in 1979, were submitted to this laboratory for testing for antibodies against Ross River antigen. These persons had been ill while in Fiji with polyarthritis and a rash; 3 of them supplied paired sera and the fourth a single, convalescent sample. The sera were tested for HI antibodies against Ross River and WEE antigens; the two samples from one of the patients were positive with a titer of 1:40 and 1:20, respectively, against Ross River antigen only. The remaining reactions were negative.

LaCrosse encephalitis in New York State (M. Grayson, R. Buck, K. Obom, C. Reed, A. Brescia, A. Main, B. Beaty, and R. Shope). A 13 year old boy from Pound Ridge, Westchester County, New York died of LaCrosse encephalitis at Yale-New Haven Hospital during August, 1978. The diagnosis was made in this laboratory on the basis of a serologic rise to LaCrosse antigen by CF test. In collaboration with the New York State Department of Health, a serosurvey of animals and persons resident in Pound Ridge as well as a limited entomological survey was carried out. Forty larvae of Aedes triseriatus were reared from a tire from the case's yard. Attempts in mice to isolate virus from adults emerging from the larvae were negative as were attempts in mosquitoes. Of 31 human sera (14 from children), LaCrosse HI and neutralizing antibody was detected in one adult and one 11 year old. Of 3 squirrel and 3 mouse sera, one squirrel was positive by neutralization test to LaCrosse virus.

In addition, paired sera from 49 patients in Connecticut who had CNS disease during the summer of 1978 were tested by HI test; 8 had antibody to Jamestown Canyon virus, 5 to LaCrosse, and 1 to trivittatus virus. Neutralization tests of the 5 LaCrosse positive sera revealed only one positive, a man who resided in Stamford which is very near Pound Ridge.

This study yielded presumptive evidence for LaCrosse encephalitis virus infection in man and rodents in the areas of Connecticut and New York, just north of New York City.

Nile rats from Sudan (G. Walker and R. Shope). HI tests were carried out on 2 Nile rat sera. The sera were from animals imported from Sudan to Colorado where they were to be used in experimental rodent control studies. The animals became sick shortly after arrival and an animal caretaken also had a febrile disease. Rift Valley fever was suspected. The HI tests of the 2 rat sera were negative for Rift Valley fever and also for Semliki Forest, chikungunya, Middelburg, Wesselsbron, dengue-2, Germiston, and Saint-Floris viruses.

V. Development of new techniques

Polyvalent spot-slides for IF tests with flaviviruses (J. Casals). Ideally such slides should react positively with all sera having antibodies for any virus in group B. Considering that there are currently over 50 flaviviruses and that, for technical reasons, slides containing more than 4×10^4 cells in a spot are unsatisfactory due to overcrowding, it follows that only a limited number of viruses can be directly represented in a slide; the use of polyvalent flavivirus slides is based on cross-reactivity among the viruses in the group.

Spot-slides were prepared containing 6 viruses representing different complexes in group B; allowing 4×10^4 cells in a spot, each virus was represented by a maximum number of 6500 infected cells. Preparation of such slides requires a great deal of effort in that the optimal cell for each virus must be determined and a close co-ordination must exist between the days when the cultures are infected with the different viruses in order that they all attain a CPE between $\pm$ and 2 plus on the same day. After preliminary explorations, the following viruses, cells and harvesting time after infection of the culture were used: JE, VERO, 3 days; Rocio, VERO, 5 days: yellow fever, VERO, 4 days; a nearly equivalent mixture of dengue 1 and 2, LLC-MK2, 7 days; and Langat, BHK-21, 5 days.

The infected cells were processed separately according to virus, the cell suspensions adjusted to a count of 4×10^6 cells per ml and mixed in equal parts thus making a suspension containing 0.8×10^6 cells/ml infected with each virus (the two dengue types are considered as one). Spot-slides are prepared, as described previously, with the cell mixture by depositing one drop from a 27-gauge needle on each spot; these drops have a volume very close to 0.01 ml. The capacity of these slides to detect antibodies against flaviviruses has been tested with hyperimmune mouse sera or ascitic fluids; the result of a test is shown in Table 21.

Four of the immune reagents tested failed to react at dilution 1:8, lowest used; it is possible that two of these reagents, Banzi and Tembusu, may have had low homologous titers. It may be advisable in the future to prepare two sub-sets of group B polyvalent slides, each with 5 or 6 different viruses, in order to increase the probability of complete coverage.

The use of micro cell cultures for neutralizing antibody assays (J. Casals). Assays for neutralizing antibodies are a real problem with viruses that produce plaques with difficulty or with which the neutralization test in mice lacks sensitivity or is delayed due to a long incubation period. Among such viruses are Congo-Crimean hemorrhagic fever (CCHF) and lymphocytic choriomeningitis (LCM).

Previous work in this laboratory (Smith et al., 1977; Tignor et al., 1980) has shown the possibility of applying a method, reduction of fluorescent foci of infection (RFFI) to CCHF and rabies; the method is based on counting foci of infection visualized by immunofluorescence, in the absence of, or prior to, plaque formation. The method is sensitive and quantitative but time consuming.

Attempts are under way to devise a simplified version of the RFFI by means of micro-cell cultures, in which the presence or absence of virus is also determined by immunofluorescence but with no attempts to count foci: a quantal response is sought rather than a quantitative one. The test may be less accurate than the RFFI but far more expeditious.

The test now being developed is carried out in a unit, Bellco micro-slide culture chamber. The unit consists of a silicon rubber block 55 x 25 x 12 mm having 10 cylindrical performations, about 5 mm in diameter at the base. A microscope slide is placed under the openings on one side of the block and a metal plate with matching holes on the other side; the whole is held together with 2 lateral clips with the result that 10 wells, each with a capacity of 0.4 ml are formed, each well becoming a culture chamber. In this laboratory teflon-coated spot-slides are used instead of plain microscope slides. Each chamber is seeded with from 1 x 10^4 to 2 x 10^4 cells in 0.3 ml of medium and the slide is ready for use in 1 or 2 days. The virus or virus-serum mixtures in 0.05 ml amounts are introduced in the wells after removing the medium; following 1 hour adsorption 0.3 ml maintenance medium is added. The unit is disassembled at the adequate time--3 to 5 days later --and the micro-cultures processed for immunofluorescence staining.

A comparison between the results of a mouse neutralization test and a neutralization of immunofluorescence (NIF) test was done with Bandia virus and immune mouse sera for Bandia, CCHF and Abu Minah viruses. Mixtures of constant virus and serum dilutions were incubated at 4C for 18 hours after which they were assayed for presence of residual virus by intraperitoneal inoculation to 3-day-old mice and by inoculation to LLC-MK2 micro-cultures. The result of the test is given in Table 22; the mouse test required 10 days to bring to completion, the NIF test only 5. In the NIF test a small amount of protection was given by the CCHF serum against Bandia virus, no protection was observed in the mouse test; additional work is required in order to determine the sensitivity and dependability of the technique.

Table 21

Immunofluorescence test with group B polyvalent slides

Antiserum	Titer	Antiserum	Titer
CETBE	1:16+	Phnom Penh Bat	0
Banzi	0	Powassan	1:16+
Dengue 1	1:16+	RSSE	1:16+
Dengue 3	1:16+	Spondweni	0
Dengue 4	1:16+	SLE	1:16+
Edge Hill	1:16+	Tembusu	0
Ilheus	1:16+	Wesselsbron	0
KFD	1:16+	West Nile	1:16+
MVE	1:16+	Yellow Fever	1:16+
Omsk HF	1:16+	Zika	1:16+

0 = reaction was negative at serum dilution 1:8, lowest used.

Table 22

Assay for neutralizing antibodies against Bandia virus. Comparison between results with a mouse neutralization test and results of a neutralization of immunofluorescence test

Serum	Mouse test* Serum dilution			NIF test# Serum dilution		
	Und.	1/4	1/16	Und.	1/4	1/16
Bandia	0	0		0	0	
CCHF	8	8	8	0	+	+
Abu Minah	8	8	8	+	+	+

* Mice dead of 8 inoculated; the titer of the virus in the inoculum was $10^{2.3}$ LD_{50}.

\# 0, indicates no virus detected in the culture; +, virus detected in the culture; the titer of the virus in the inoculum was 10^2 immunofluorescent doses (IFD).

Single inoculation immune sera for typing Crimean Congo Hemorrhagic Fever, Hazara and Dugbe viruses using the indirect immunofluorescence assay (J. D. Converse). Recent investigations of Casals and Tignor have demonstrated antigenic relationships of Crimean-Congo Hemorrhagic Fever (CCHF) virus with member viruses included under "Bunyavirus-like" in the family Bunyaviridae. Application of immunofluorescence, HI, and inhibition of fluorescent foci showed antigenic associations between CCHF, Hazara (HAZ), Nairobi sheep disease (NSD), Dugbe (DUG), and Ganjam (GAN) viruses. In the above studies hyperimmune mouse serum or ascites fluid antibodies were utilized in the tests. The present study extends these studies by comparing one-injection immune sera prepared in hamster and mouse using CCHF, HAZ, and DUG viruses as antigens with multiple injection immune serum for each of these same viruses.

One injection sera of hamsters have been shown previously to be specific by complement-fixation test (CF) among the California group arboviruses (Sprance, H.E. and R.E.Shope, 1977. Amer. J. Trop. Med. Hyg., 26: 544). In the present study, female golden hamsters (3-4 week old) and female white mice (5-6 week old) from the Taconic Farms colony were immunized with 5000 suckling mouse LD_{50} of virus in phosphate buffered saline by either the intraperitoneal (mice) or subcutaneous (hamster) route. All animals were anesthetized and bled by cardiac puncture 10-day post inoculation. Prior to examination in reciprocal indirect immunofluorescence assays (IFA) each individual animal serum was extracted with acetone and ethyl ether according to the method described by Casals and Tignor (Proc. Soc. Exp. Biol. Med., 1974, 145: 960) and tested by employing spot-slides prepared with CER cell cultures (3×10^6 cells/ml) infected with CCHF, HAZ, or DUG viruses.

The results are summarized in Table 23. Almost all individual single inoculation mouse and hamster serum were specific for their respective immunizing virus. Slight cross reactions were noted with

Table 23

Indirect immunofluorescence spot-slide assay of mouse and hamster serum

Serum Virus antigen	Mouse number	FA-IgG/FA-IgM Titer[a] Congo	Hazara	Dugbe	Hamster number	FA-IgG Titer[a] Congo	Hazara	Dugbe
Congo	1	128[b]/64	0/0	0/0	1	160	0	0
	2	128/64	0/0	0/0	2	80	0	0
	3	64/16	0/0	0/0	3	80	0	0
	4	128/32	0/0	0/0	4	80	0	0
	5	64/64	0/0	0/0	5	0	0	0
	6	32/64	0/0	0/0	6	80	0	0
	7	32/128	0/0	0/0	7	20	0	0
Hazara	1	0/0	32/0	0/0	1	5	160	0
	2	0/0	32/4	0/0	2	5	160	0
	3	0/0	32/4	0/0	3	5	160	0
	4	0/0	64+/8	0/0	4	5	160	0
	5	0/0	64/8	0/0	5	5	160	0
	6	0/0	128+/8	0/0	6	5	320	0
	7	0/0	64/16+	0/0	7	5	80	0
Dugbe	1	4/0	0/0	32/NS[c]	1	0	0	80
	2	4/0	0/0	64+/NS	2	0	0	160
	3	4/0	0/0	64/NS	3	0	0	80
	4	4/0	0/0	64+/NS	4	0	0	0
	5	4/0	0/0	0/0	5	0	0	80
	6	4/0	0/0	0/0	6	0	0	80
					7	0	0	0
Hyperimmune serum (Casals)								
Congo		256		8				
Hazara		32	10+	4				
Dugbe		16		16				

[a]Fluorescein conjugates (Cappell Laboratories, Cochranville, Pa.): Goat antimouse-IgG, Goat antimouse-IgM (Mu-chain specific), Rabbit antihamster-IgG (1:15 dilution, 15 µl/spot).
[b]Reciprocal of serum titer: serum dilution, 2 fold.
[c]NS, nonspecific reaction.

[illegible] cells, however, [illegible] (1:4 for [illegible] and 1:5 for [illegible]) [illegible] difficulty. [illegible] tested, 3 of 21 (14.3%) [illegible] were found with no detectable [illegible].

Homologous virus IgG antibody titers were higher [illegible] than those found with [illegible] sera. [illegible] of 7 (5 of 7) of the mice injected with CCHF or [illegible] titer of 1:80 or greater, while 86% (6 of 7) injected with [illegible] IgG titer of 1:160 or greater.

In the [illegible], CCHF and [illegible] single [illegible] did not show the cross reactions previously reported to be [illegible] hyperimmune sera. However, [illegible] single injection [illegible] all mice did show cross reactions at the lowest dilution tested (as mentioned above). When analyzing the IgG and IgM antibody response of these mice, it was noted that IgM titers were lower than the co-existing IgG titer, with the exception of 3 animals, i.e., 3 of 7 mice inoculated with CCHF virus. The non-specific CA reactions noted for the [illegible] mouse IgM antibody are unexplained and these sera are to be retested at a later date.

In addition, all the above antisera and their respective virus antigens are presently being evaluated in enzyme immunoassay (EIA-EA) and radioimmunoassay (RIA) systems for their possible use in rapid diagnostic procedures for CCHF, HAZ and DUG viruses.

Isolation of anti-viral antibodies by immunoadsorbent chromatography (J.D. Converse). Immunoadsorbents allow the use of the interaction of antigens with their antibodies to purify specific, active antibodies or antigens in high yield from dilute or small volume samples. The desired immunoadsorbent is one having high adsorbent capacities with good chemical stability so as to allow regeneration of the immunoadsorbent for re-use many times. The coupling of intact virus or virus antigens to agarose for the purpose of isolating virus-specific antibodies from serum or immunoglobulin preparations have been of recent interest (Lecomte, J. and D.A.J. Tyrrell, 1976. J. Immunol. Meth. 13: 355; Borriss, E. and G. Koch, 1975. Virology 67: 356; Hazlett, D.T.G., 1977. Immunochem., 14: 473). The investigated model of our studies has been to examine the feasibility of coupling sucrose-acetone extracted infected mouse brain suspensions (BRSAFD-10%) of Congo (CCHF), Hazara (HAZ) and Dugbe (DUG) viruses to Sepharose 4B (Pharmacia, Inc.) using the cyanogen bromide activation method (CNBr-Seph 4B) described in recent reports (Porath, J., et al., 1967. Nature 215: 1491; Cautrecases, P. and C.B. Anfinsen, 1971. Ann. Rev. Biochem. 40: 259; Separation News No. 3, 1979, Pharmacia Fine Chemicals).

Briefly, the CNBr-activated Sepharose 4B prepared in the laboratory (or from commercial sources) was washed with coupling buffer and transferred to a solution of protein, i.e. BRSAFD-10%, in the same buffer. Protein concentrations, 15 to 32 mg (Lowry procedure) were used for binding to the CNBr-Seph 4B. The gel-protein suspension was coupled by mixing together end-over-end for 16-24 hours at 4°C. After coupling, any remaining reactive groups were blocked using 0.2M glycine, pH 8.5 or 1M ethanolamine pH 8.0. The virus antigen-gel was then extensively washed using buffers of alternating high and low pH to remove all non-covalently bound proteins.

The chromatographic stage began by packing the gel into a column (usually a 10 ml disposable syringe body) followed by stabilization with 2-3 column volumes of starting buffer, 0.1 M phosphate buffer, pH 7.0 containing 0.25M NaCl, for optimal binding between the immunoadsorbent and antibodies of the various samples. The antibody-containing sample was then transferred to the starting buffer, usually a 1:2 or 1:4 dilution, and applied to the gel and incubated from 30 to 60 minutes at room temperature or in the cold at 4°C. The column was subsequently washed with 2-3 volumes of buffer to remove unbound material. Next several eluent buffers were used to desorb the bound antibodies from the immunoadsorbent. The flow rate of 1/2 ml/minute was used, fractions collected and optical density at 280 nm recorded to determine the protein concentration. After desorption protein fractions were returned to neutrality and desalted on Sephadex G-25 (Pharmacia, column PD-10), concentrated by negative-pressure dialysis and examined by immuno-gel diffusion analysis (IGDA) and indirect-immunofluorescent assay (IFA) using spot-slides prepared with cell cultures infected with CCHF, HAZ and DUG viruses. Examination with these eluted fractions using the enzyme-immunoassay (ELISA) and radioimmunoassay (RIA) techniques are in progress.

In our initial experiments ^{3}H-labelled CVS (rabies) virus was utilized to test for the conjugation of virus antigen to CNBr-Seph 4B, Table 24 . Approximately 22% of the total ^{3}H-CVS viral protein bound to the activated agarose. In the subsequent experiments using BRSAFD-10% suspensions of CCHF, HAZ and DUG, 44.81%, 51.53%, and 33.64% of the total protein bound to the CNBr-Seph 4B, respectively. It should be pointed out however, that not all of the bound protein is thought to be virus protein. Prior to adsorption and sequential elution of anti-virus antibody from these columns mock elution with the three elution buffers (20 ml each) were run, i.e., 0.05M Tris buffer. The supernatant eluent from each preparation was assayed for additional unbound protein and subtracted from the original protein concentration used, and the final percent protein bound calculated (column #3, Table 24).

Next, a 1-2 ml sample of immune mouse ascites fluid or serum, previously acetone-ethyl ether extracted and concentrated to 1/5th the original volume was placed on the respective virus-immunoadsorbent column, incubated for 60 minutes at room temperature and sequentially

eluted with the above buffers. Fractions were collected and later pooled according to the elution buffer used. Several samples were run on their respective columns and pools from several runs combined for use in IGDA and IFA analysis. As shown in Table 24, antibody to each respective virus was detected by FA analysis in the first eluent buffer, i.e., 0.05M Tris, but nonewas found with the chaotropic ion buffer. In IGDA analysis with specific rabbit antimouse-IgG and IgM these antibodies were shown to be of the IgG immunoglobulin class. No other serum proteins were detected. Antibody was also shown in the phosphate buffered saline wash prior to sequential buffer elution indicating that all antibodies in these samples had not bound during the incubation period. Repetitive adsorption using the phosphate buffered wash suspensions was not attempted. In IFA studies, using reciprocal patterns of the eluted antiserum fractions against the homologous viruses, no FA cross-reactions were noted as were found with untreated fluids (i.e., not passaged through the immunoadsorbent column) studied in the similar manner. Studies are still in progress.

Enzyme-immunoassay (ELISA) antigen production: Microcarrier cell culture system and virus production (J.D. Converse). In earlier cell culture studies, using 150 cm^2 plastic cell culture flasks and 490 cm^2 roller bottles in the production of arbovirus antigen for use in enzyme-immunoassay (ELISA) of antibody for different arboviruses, it was found that usually low yields, or less than adequate amounts of material were obtained for test purposes. Mass production of cell cultures and virus production using these containers met with limited success. The methods initially used were also laborious, expensive and in all cases presented significant biohazard problems in the handling of large volumes of infectious materials. Our interest was in techniques for large scale production of cells, and ultimately virus production, with less cumbersome cell manipulation; in addition to reduced biohazards of multiple handling of the infectious material we aimed to achieve high concentrations of virus with fewer purification steps.

Recently, cell cultures on beaded microspheres have been developed with several mammalian cell cultures (Giard, D., et al., 1977. Appl. Environ. Microb. 34: 668). These successes have aroused our interest in the possible application of this technique for the production of quantities of cells, and subsequently for the use in production of quantities of arboviruses for use as antigen in ELISA, radioimmunoassay (RIA), and other serological applications.

Microcarriers are polyacrylamide spheres approximately 150 micron in diameter providing support for growing of attachment-dependent cells in a suspension culture. These specially treated beads have optimized cell culture systems for routine production of large quantities of cells, as well as help fulfill some of the prerequisites mentioned above.

Table 24

Protein concentration of virus-immunoadsorbent columns and antibody activity of eluted fractions of mouse anti-virus antibodies over the immunoadsorbent columns.

Antigen	Protein conc. used	% Protein bound to column Before	After[a]	Assay used	PBS wash	0.05M Tris	1M NaCSN	3M NaCSN
^{3}H-labelled CVS virus	50 1 sample = 3.31 x 10^5 cpm[b]	21.44%	----------Not tested----------					
CCHF-BRSAFD IbAr 10200 C-1127 5-8-74	15.21 mg	54.14%	44.81%	IGDA[c]	+	+	-	-
				IFA[d]	+	+	-	-
Hazara-BRSAFD JC-280 1-26-72	31.3 mg	56.90%	51.53%	IGDA	+	+	-	-
				IFA	+	+	-	-
Dugbe-BRSAFD IbAr 2484 C-674 5-18-66	15.66 mg	41.38%	33.64%	IGDA	+	+	-	-
				IFA	+	+	-	-

[a] Percent protein bound to CNBr-Sepharose 4B column before and after mock elution with elution buffers.

[b] Counts per minute radioactivity, Beckman LS-150 liquid scintilation system. The ^{3}H-CVS virus kindly provided by Dr. G. Tignor, YARU.

[c] IGDA, immuno-gel diffusion analysis.

[d] IFA, indirect immunofluorescent assay.

In our studies the initiation of microcarrier cultures was as prescribed by the manufacture instructions (BIO-RAD, Biocarriers). Briefly, microcarrier beads were suspended in 50 mM HEPES - 0.15M NaCl buffer adjusted to pH 6.4, to a concentration of 20 g/liter. This suspension was sterilized by autoclaving 121°C for 20 minutes and stored sterile at 4°C. Usually 500 mg to 1 g microcarrier per 250 ml glass spinner bottle culture was prepared. From monolayer cell cultures of CER, LLCMK-2, XTC-2 (Xenopus laevis), and $C^{6/36}$ clone of Singh's Aedes albopictus, 2-3 x 10^7 cells suspended in complete medium (minimal essential medium, Hanks with 10% fetal calf serum for mammalian cell culture; Leibovitz, L-15 medium with 10% fetal calf serum plus 10% tryptose broth for invertebrate cell culture) was mixed with dispersed microcarrier beads and the total volume brought to 100 ml in the 250 ml spinner flask. The flask was placed on a non-heating magnetic stirrer at 75 rpm, the mammalian spinner cultures maintained at 37°C, the invertebrate spinner cultures at 30°C. Growth medium was changed approximately every 48 hours, the pH of the medium was monitored and cell counts made daily. In virus production studies the microcarrier cell cultures, usually 1.65 $\pm$ .64 x 10^6 cells/ml (average), were infected with either Congo, Hazara, Dugbe or Ross River virus stocks of 10% homogenate of infant suckling mouse brain at selected multiplicities of infection. Standard procedures were followed for the trypsinization of cells, cell counts, recovery of supernatants for virus assay and the preparation of spot slides. The rate of virus antigen development was assayed by indirect immunofluorescent antibody technique (IFA) modified from the method described by Igarashi (J. gen. Virol. 1978. 40: 531), i.e., in our tests we used 1.5 x 10^6 cells/ml to prepare drops for microscope spot slides. Also, 15 µl/spot of specific mouse or hamster immune serum or immune ascites fluid were used. All slides were stained with 1:15 dilution of commercial fluorescein-conjugated anti-gamma globulin (Cappel Laboratories, Cochranville, PA).

Virus titration assay using VERO or CER cells was by the fluorescent focus assay technique (FFU) as described by Digoutte, et al. (1976 Ann. Microbiol. Inst. Pasteur. 127B: 573) using 4 chamber Lab-Tek slides (Miles Laboratories, Inc., Naperville, Ill.) Fluorescent foci of Ross River, Dugbe and Congo viruses were read at 48 hours, 72 hours, and 96 hours, respectively.

The microcarrier growth curve for CER cell culture is shown in Figure 1. Each point represents the mean of 4 similar microcarrier experiments, and the 95% confidence limits of the mean is denoted. The dash curved line represents the bivariate data logarithmic transform curve for prediction analysis of cell population at any time interval.

Cell density for CER cells ranged from 1.36 x 10^6 to 4.6 x 10^6 cells/ml (1.36 x 10^8 to 4.6 x 10^8 cells per 100 ml) with a mean of 2.30 $\pm$ 1.57 x 10^6 cells/ml by 264 hours (11 day). In all studies, complete monolayers were formed on 95-100% of the microcarrier beads by day 6 (i.e., 1.2 x 10^6 to 2.43 x 10^6 cells/ml, mean = 1.82 $\pm$ 0.87 x 10^6

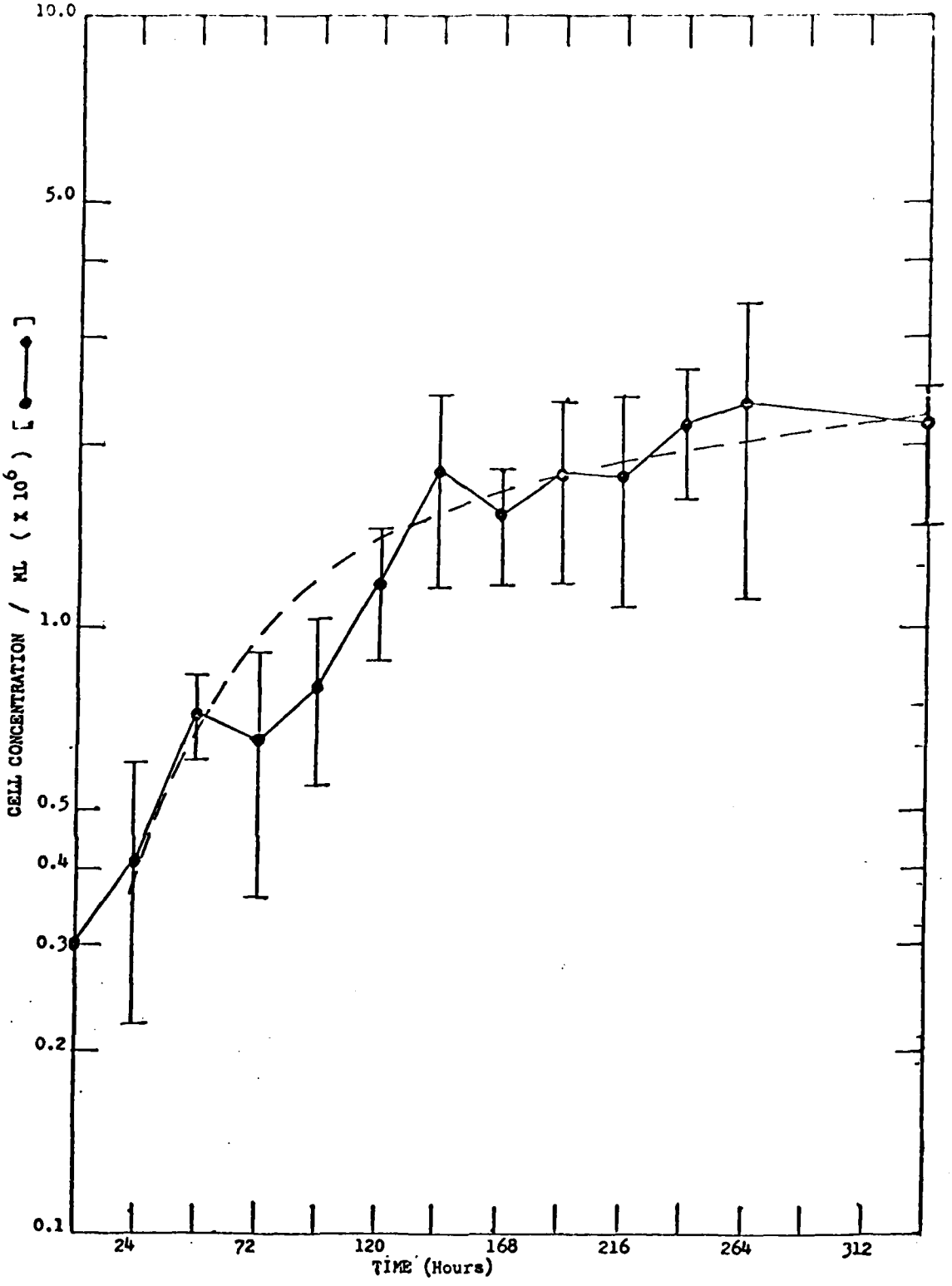


Figure 1. Growth curve of CER cell culture on microcarrier spheres. Cells were seeded at 3 x 10^5/ml in 100 ml volumes, with a microcarrier concentration of 5 mg/ml. Medium was replenished every 48 hours by removing 50 ml of spent medium, and replacing with 50 ml fresh medium. Each point represents the mean of four experiments, the 95% conficence limits of the mean is denoted. Dash curve represents the bivariate data logarithmic transform curve for prediction analysis of cell population with time.

cells/ml). The rate of growth for CER cells showed a lag period before logarithmic growth of approximately 24 hours, with a doubling time of approximately 48 hours.

Studies of CER and XTC-2 cell growth and virus production are shown in Figures 2-4. The infectious virus yield, reported as log FFU/ml, from CER cells infected with Congo virus at a multiplicity of infection of .004 is shown in Figure 2 (insert); an increase of approximately 10 fold was found. The optimal virus growth reach a peak at 4 day post inoculation (p.i.), thereafter dropping on day 5 p.i. and remaining constant through day 8 p.i., at which time the experiment was terminated. The rate of antigen development in the CER cells showed approximately 7% of the cells antigen positive by day 4 p.i., and 37% antigen positive by day 8 p.i.. The results indicate that probably little infectious virus and substantial defective virus is produced by the CER cells. Throughout the infection period no detectable cytopathic effect or cell detachment from the microcarrier beads was observed. Infectious supernatant fluids were saved, filtered thru 0.2 micron filters and stored at -20°C, also infected cells were frozen and thawed three times, centrifuged at 2000 rpm to remove cellular debris and microcarrier bead, the supernatant fluid saved separately from the above infectious fluids and also stored at -20°C for later processing as antigen for ELISA tests.

Figure 3 (and insert), show cell growth and infective virus yield with Dugbe virus at a multiplicity of infection of 2. After an initial lag period of 24 hours p.i. the infectious virus yield increased 100 fold by day 3 p.i. On the 4th day p.i. over 50% of the cells were infected with this virus. At this time time microcarrier experiment was discontinued and the Dugbe-infected cell-microcarrier suspension centrifuged at 1000 rpm for 10 minutes, the spent medium removed and the infected cell-microcarrier suspension washed three times with Hanks' balanced salt solution. The final suspension was resuspended in 50 ml phosphate buffered saline (PBS) and divided into five equal aliquots, recentrifuged, the PBS removed and the infected cell-microcarrier treated with 10 ml of one of the following fixatives; 1% glutaraldehyde, methyl alcohol, 10% formaldehyde, neutral formaldehyde, or acetone, for 30 minutes. Next followed recentrifugation and final resuspension in 3 ml of PBS-veronal buffer, pH 7.6. Cell attachment to microcarrier beads was lost using all fixatives with the exception of 1% glutaraldehyde and methyl alcohol. These latter treated beads were utilized in a standard ELISA test as antigen source (standardized at 1000 beads per reaction well) and mixed with homologous immune serum dilutions. These preliminary studies proved negative due to non-specific reactions with normal serum controls.

In IFA tests using the Dugbe-infected cell-microcarrier beads, a sample of glutaraldehyde fixed beads was mixed with serial dilutions of homologous and heterologous immune serum, plus normal serum and PBS controls. The standard IFA procedure was followed and after the

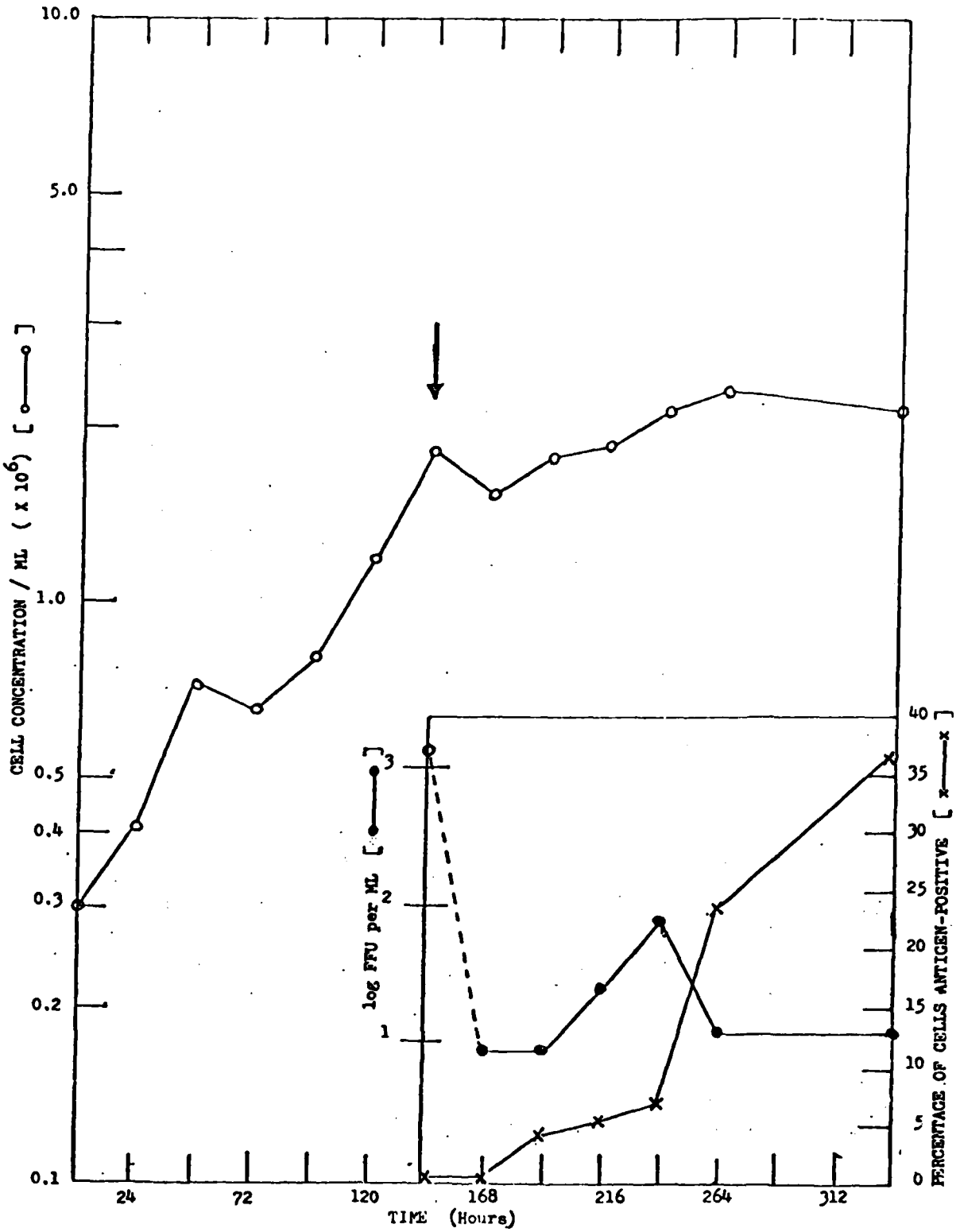


Figure 2. CER cell growth and infectious Congo virus yield with rate of Congo virus antigen development (insert) in inoculated microcarrier cell culture. Arrow denotes time of inoculation.

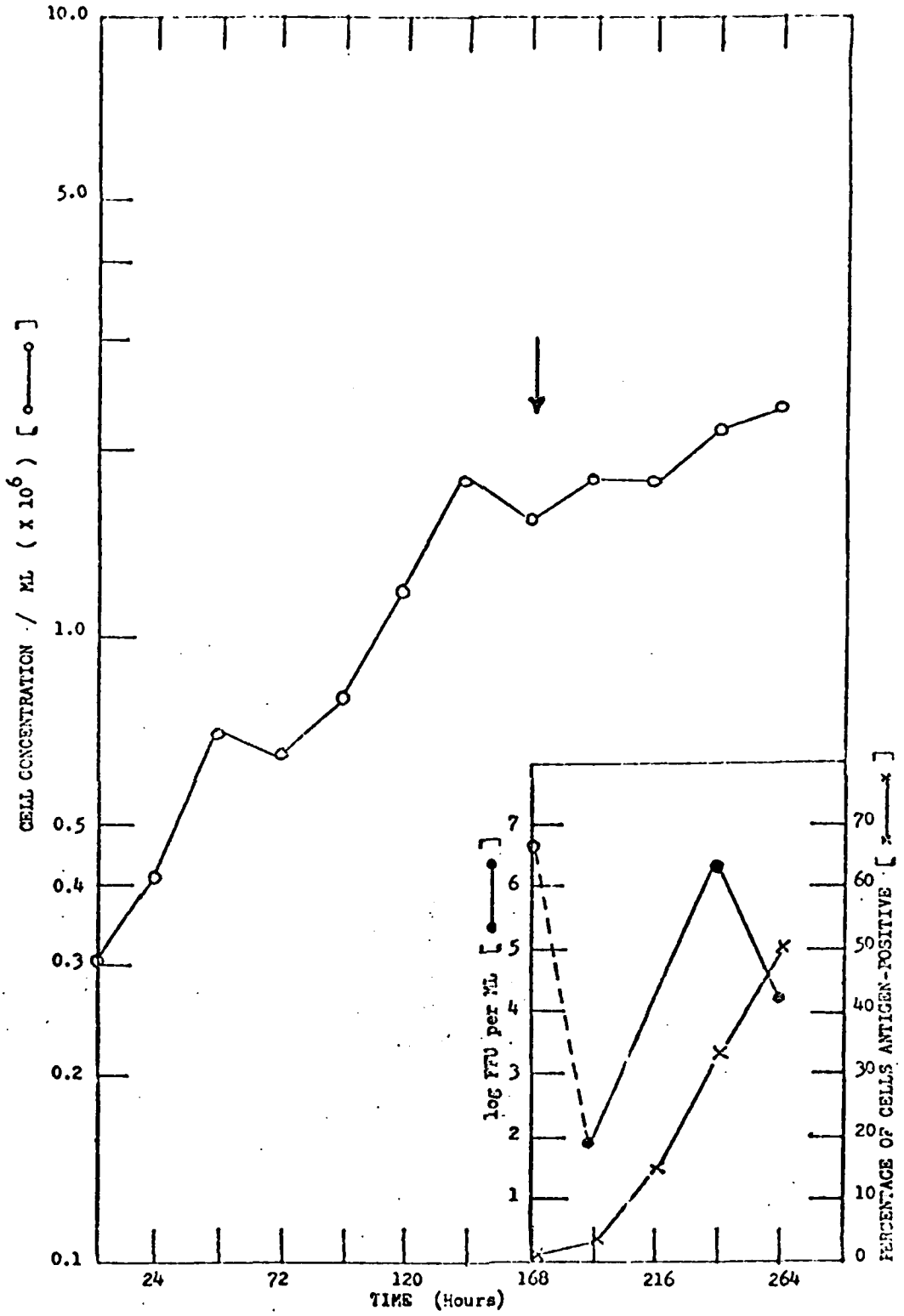


Figure 3. CER cell growth and infectious Dugbe virus yield with rate of Dugbe virus antigen development (insert) in inoculated microcarrier cell culture. Arrow denotes time of inoculation.

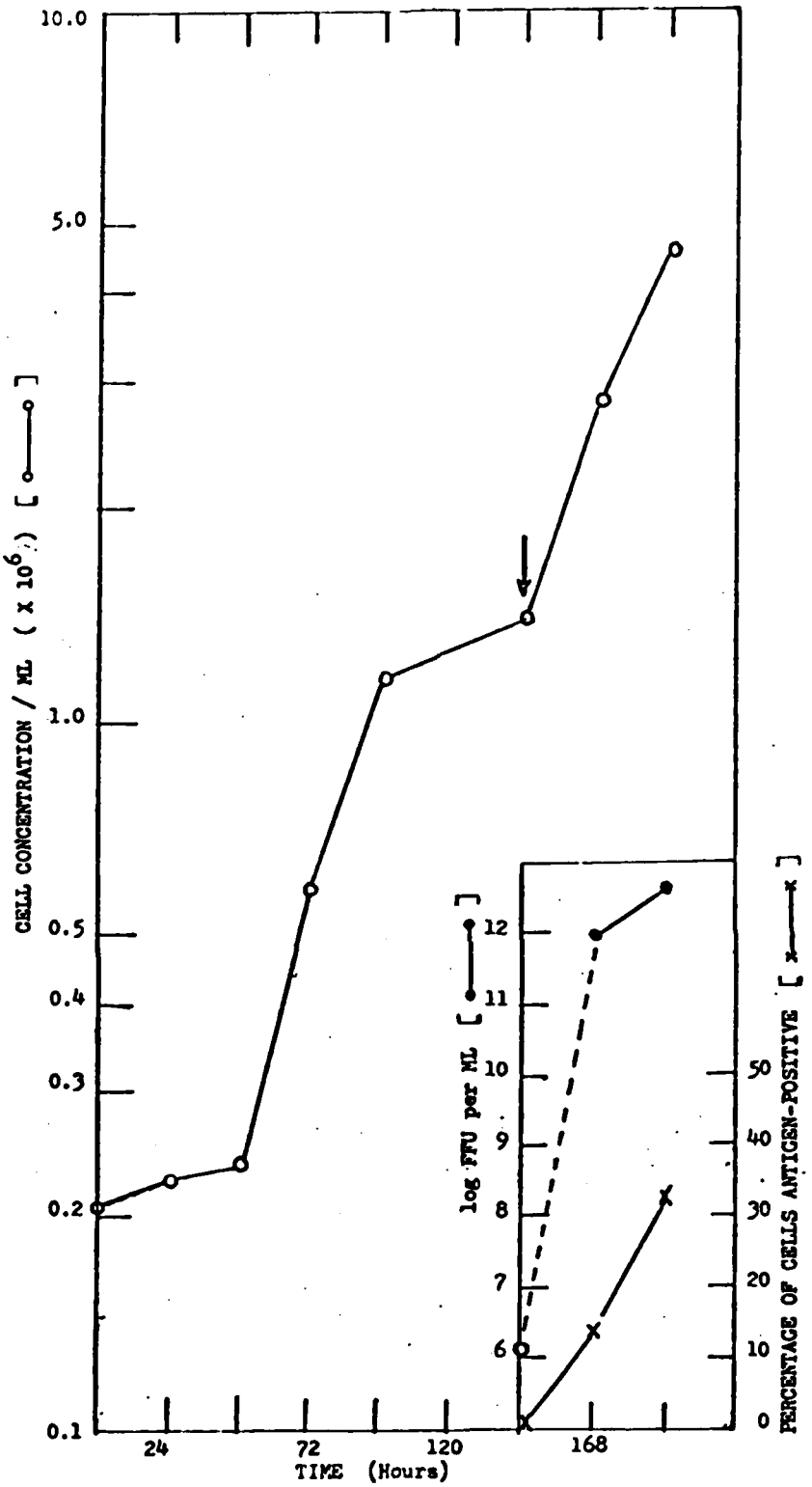


Figure 4. Growth of XTC-2 (Xenopus laevis) cell culture on microcarrier spheres. Cells were seeded at 2 x 10^5/ml in 100 ml volumes, with a microcarrier concentration of 5 mg/ml. Infectious Ross River virus yield with rate of Ross River virus antigen development (insert). Arrow denotes time of inoculation.

final wash the beads were resuspended in 15 µl of distilled water and placed on spot slides and observed in the wet state under the UV microscope. The results shown in Table 25 indicate that the glutaraldehyde treated infected cell-microcarrier beads may possibly be used in assay of homologous serum antibodes. Further studies are still in progress.

Table 25

Indirect immunofluorescent antibody assay for Dugbe serum antibodies using glutaraldehyde (1%) treated Dugbe-CER infected cell-microcarrier beads.

Antigen	Immune Serum	IFA Reaction
Microcarrier beads w/o cells	All below 1:4	negative
Uninfected CER-microcarriers	All below 1:4	negative
Dugbe-CER-microcarriers	Dugbe (1:4-1:128)	+ (1:32)
Dugbe-CER-microcarriers	Congo (1:10)	±
Dugbe-CER-microcarriers	Hazara (1:5)	±
Dugbe-CER-microcarriers	Norm. serum (1:10)	negative
Dugbe-CER-microcarriers	PBS control	negative

Figure 4 illustrates the growth curve of XTC-2 (Xenopus laevis) cell culture on microcarrier beads. The initial total cells seeded was 2×10^7 cells. After 4 days incubation at 30°C, in spinner culture at 75 rpm, over 95% of the microcarrier beads were confluent monolayers. Cell density was 1.24×10^6 cells per ml by 96 hours (4 day); the rate of growth of XTC-2 showed doubling time of 24 hours. By day 8 cell density increased to 4.59×10^6 cells/ml. On day 6 the XTC-2 microcarrier culture was infected with Ross River virus (multiplicity of infection of 0.5). Within 24 hours p.i. the XTC-2 cells began detachment from the microcarrier beads, by the 2nd day p.i. <40% of the beads remained covered with cells attached. However, in the same period a 0.5 log increase in cell growth was noted with a 400 fold increase of infectious virus. Over 36% of the cells in culture at 2 day p.i. were antigen-positive when tested using mouse immune serum to Ross River virus.

After the cells began to detach the microcarrier experiment was discontinued, the infectious supernatant saved, the cell-microcarrier mixture aspirated to effect detachment of remaining attached cells and centrifuged at 700 rpm for 20 minutes to sediment the beads. One ml of the XTC-2 infected cell suspension, i.e., approximately 9.0×10^5 cells/ml, was transferred to a single 75 cm^2 culture flask with 15 ml complete medium and incubated at 30°C. A confluent monolayer was established from these cells within 60 hours. Supernatant fluids

from this culture were recovered every 5th day thereafter, and fresh complete medium (15 ml) added to the flask. Virus production continued after 10- and 15-day p.i. The titer of these Ross River virus supernatants in VERO cells was 1×10^5 and 1×10^6 $TCID_{50}$ per ml, respectively. The XTC-2 cell culture remains confluent with mild cytopathic effect throughout the monolayer after 30 day p.i. at 30°C. Cell culture supernatant fluids will be saved and tested for use as antigen source in ELISA, RIA and other serological assays.

Adaptation of phlebotomus fever group viruses to Vero cells and pathogenicity for mice (J. Meegan and R. Shope). In preparation for study of the proteins of phlebotomus fever viruses, the relationship of proteins to serologic reactions and especially cross-reactivity with Rift Valley fever, and (in collaboration with Dr. D.H.L. Bishop, University of Alabama) study of pathogenicity markers of cloned viruses and their reassortants, selected viruses have been adapted for growth and plaquing in Vero cells and titered in Vero cells, 9 day old and in 4 week old mice by the i.c. and i.p. routes. As shown in Table 26 Punta Toro, Sicilian, and Arumowot kill 4-week-old mice by the i.c. route. None of the 12 viruses so far tested kill 4-week-old mice by the intraperitoneal route.

Although reassortant viruses are not yet available from the University of Alabama, should Punta Toro-Karimabad reassortants be produced it presumably will be feasible to determine the segment responsible for i.c. pathogenicity, based on results with wild type cloned stocks of these two viruses (Table 26).

Studies with the in vitro feeding of ticks on meals in glass capillaries (T. Aitken and A. Messer). Previously a glass capillary technique was developed in this laboratory for feeding mosquitoes artificially which permitted demonstrating virus transmission capability of infected mosquitoes (Aitken, Mosq. News, 37: 130, 1977).

It was hoped that the above method might be adapted for use with ticks as it was known that Burgdorfer had described a technique for infecting ticks with pathogens involving the use of glass capillaries (J. Infect. Dis., 100: 212, 1957). Burgdorfer worked with hard (ixodid) ticks, immobilizing them in plasticine, after which the capillary tip could be inserted readily over the mouthparts, and the tick proceeded to feed. The method worked in our hands with Amblyomma americanum because an unengorged hard tick is flat, but when it was tried with the soft tick, Ornithodoros moubata, it failed. The tick could not be immobilized because the body is more rounded and mattress-like; the tick continually inflated and deflated itself and quickly squirmed loose. The problem was finally resolved by applying the dorsum of the tick to a piece of masking tape which in turn was stuck to a glass slide. From the anterior position, the glass capillary tip was inserted more or less horizontally over the mouthparts (chelicerae and hypostome) of the ventrally-presenting tick; the capillary was then allowed to rest on (or firmed into) a small mound of plasticine.

Table 26

Pathogenicity of selected viruses of the phlebotomus fever group for mice

Virus	p.f.u. in Vero	9 day old ic	9 day old ip	4 week old ic	4 week old ip
Punta Toro (UAB cloned)	4×10^7	7.3[a]	-[b]	7.9	-
Karimabad (UAB cloned)	7×10^6	-	-	-	-
Punta Toro (YARU)	1.8×10^6			7.0	-
Icoaraci	3×10^4	+[c]	+	-	-
Candiru	1.8×10^5	+	-	±[d]	-
Frijoles	4×10^7	±	-	-	-
Sicilian	1.2×10^6	±	+	+	-
Anhanga	5×10^5			-	-
Itaporanga	3×10^5	±	-	-	-
Chagres	5×10^5			-	-
Saint-Floris	4.5×10^6			-	-
Arumowot	5×10^5			+	-
Nique	1.8×10^6			-	-

[a] log LD_{50}/ml

[b] negative in undiluted inoculum

[c] all mice receiving undiluted inoculum died

[d] some but not all mice receiving undiluted inoculum died.

Care was taken to jam firmly the capillary tip against the tick otherwise the tick was able to work the capitulum loose.

In this manner 4 female Ornithodoros were exposed to artificial meals. The meal for 2 of the ticks consisted solely of 10% fetal bovine serum and for the other ticks 10^{-3} adenosine triphosphate (ATP) was added. After one hour, the ticks feeding on the latter meal (ca. 0.02 ml) had almost exhausted it, whereas those ticks deprived of the ATP had barely taken any meal even by 2 hours; when ATP was added to the meal subsequently, the ticks fed more readily.

As in the case of the earlier mosquito studies, such meals exposed to feeding virus-infected ticks could subsequently be assayed for infectivity in a tissue culture system or else inoculated into clean mosquitoes and after a suitable incubation period, processed for infectivity by fluorescent microscopy.

In the course of this study, additional work was done with Amblyomma americanum and the Burgdorfer technique modified. This flat tick was readily immobilized on its back in a small pyramid of plasticine affixed to a glass slide. Capillaries, charged with 0.02 ml of meal (10% FBS in PBS and antibiotics, penicillin and streptomycin), were introduced vertically over the mouthparts of the recumbent tick. Each capillary was held in position by a tab of strengthened adhesive tape projecting from a test tube rack and bearing a small hole which held the capillary in position. To avoid jiggling of the tabs (and thus disconnecting the capillary from the ticks), a small rubber cork (bored) was placed over the capillary and rested on the tab; the cork provided the necessary weight to prevent movement.

Using this technique, 17 Amblyomma females were successfully fed over a period of 2.5 hours. Nine ticks imbibed the full amount of the meal (0.02 ml), 4 ticks took 0.015 ml and 4 ticks took 0.01 ml.

It would thus seem that these techniques might well be used in the study of tick-borne viruses.

Starch-gel electrophoresis of invertebrate cell line isozymes (W.J. Tabachnick and D.L. Knudson). With the increasing number of established invertebrate cell lines, the need for criteria for characterization and identity becomes apparent. The usefulness of isozyme profile analyses as criteria for characterization and identification of fourteen lepidopteran and two dipteran cell lines was examined. Eighteen isozyme systems were evaluated for their discriminating ability. The sixteen cell lines and the eighteen isozyme systems provide a stringent test of the ability of the isozyme profile analyses to discriminate between taxonomic relatives.

Table27 lists the sixteen invertebrate cell lines which were analyzed for enzyme phenotypes. The samples were stored at -70°C,

Table 27

Invertebrate Cell Lines

ORDER	FAMILY	GENUS SPECIES	COMMON NAME	CELL LINE DESIGNATION	ASSIGNED NUMBER*
Diptera	Culicidae	Aedes aegypti	yellow fever mosquito	ATC-10	1
		Aedes albopictus	mosquito	ATC-15	2
Lepidoptera	Arctiidae	Estigmene acrea	salt marsh caterpillar	BTI-EAA	3
	Bombycidae	Bombyx mori	silkworm	BM-N	4
	Lasiocampidae	Malacosoma disstria	forest tent caterpillar	IPRI-MD-108	5
	Lymantriidae	Lymantria dispar	gypsy moth	IPLB-LD-65Z	6
	Noctuidae	Heliothis zea	corn earworm or cotton bollworm	IMC-HZ-1	7
		Heliothis zea	corn earworm or co-ton bollworm	IPLB-HZ-1075	8
		Mamestra brassicae	cabbage moth	IZD-MB-0503	9
		Spodoptera frugiperda	fall armyworm	IPLB-SF-21AE	10
		Spodoptera littoralis	cotton leafworm	UIV-SL-573	11
		Trichoplusia ni	cabbage looper	TN-368	12
		Laspeyresia pomonella	codling moth	CP-1268	13
		Laspeyresia pomonella	codling moth	CP-169	14
		Manducu sexta	tobacco hornworm	MRRL-CH-I	15
		Choristoneura fumiferana	spruce budworm	IPRI-CF-124	16

All cell lines grown in TC100 medium, except ATC-10 and ATC-15 which were grown in Mitsuhashi and Maramorosch medium.

*The assigned number represents an arbitrary designation.

and their enzyme activity was stable under these conditions for ten of the enzymes tested.

The enzymes tested and their abbreviations are listed in Table 28 . Eight enzymes proved unsuitable because the profiles were either poorly resolved or there was a lack of enzymatic activity in the majority of cell lines. Nevertheless, ten enzymes consistently yielded reproducible profiles. Esterase (EST) profiles contained many bands of varying intensity, and as a result of this variation, EST proved to be the least useful enzyme for diagnostic comparisons. Although G6PDH and LDH profiles were recordable, the smeared profiles were suggestive of some enzyme denaturation due to the freeze-thaw treatment of the cell sample. Nevertheless, they were useful for a number of cell lines.

The relative mobilities of the enzyme phenotypes that were observed are recorded in Table 29. These data reveal that the cell lines were distinguishable from each other with respect to the mobility of their enzymes. The IPLB-LD-65Z and IPLB-HZ-1075 lines are exceptions because they were not distinguishable from TN-368 and IPLB-SF-21AE respectively. Initially, the BTI-EAA was not distinct from TN-368. A second BTI-EAA culture was requested from the original supplier of the line, and it yielded the relative mobilities as recorded (Table 29).

Although the cell lines are distinguishable, only three cell lines were tested and shown to exhibit enzyme mobilities consistent with the species of origin. Adult Ae. aegypti and Ae. albopictus mosquitoes and Bombyx mori larvae yielded enzyme phenotypes analogous to those observed for the ATC-10, ATC-15, and BM-N cell lines respectively.

These data (Table 29) also suggest that the screening of a large number of enzymes may not be necessary to distinguish the cell lines. For example, the enzyme system of IDH, ME, PGI, and PGM discriminate between the cell lines in this study.

Since it was demonstrated that the BTI-EAA cell line was either inadvertently contaminated with TN-368 or a TN-368 flask was mislabeled as BTI-EAA in this laboratory, the usefulness of the isozyme approach for identification was emphasized. Since a number of invertebrate cell lines are maintained in many laboratories, there is clearly the possibility of an error in labeling or in contamination. The isozyme procedure provides criteria by which these mistakes could be detected.

The enzyme phenotype should not be considered as the sole criterion for identity, but rather as an adjunct to cell morphology, growth characteristics, and karyology. The IPLB-LD-65Z and IPLB-HZ-1075 cell lines, for example, exhibit parameters identical to the TN-368 and IPLB-SF-21AE cell lines, and thus, they probably resulted from laboratory accidents in handling. The enzyme phenotypes for the true IPLB-LD-65Z and IPLB-HZ-1075 cell lines remain to be demonstrated.

Table 28

Enzymes Tested

SUITABILITY FOR TEST	ENZYME	ENZYME COMMISSION NUMBER	ENZYME ABBREVIATION
Suitable			
	Esterase	3.1.1.2	EST
	Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH
	Hexokinase	2.7.1.1	HK
	Isocitrate dehydrogenase	1.1.1.42	IDH
	Lactic dehydrogenase	1.1.1.28	LDH
	Leucine amino peptidase	3.4.1.1	LAP
	Malic enzyme	1.1.1.40	ME
	Phosphoglucoisomerase	5.3.1.9	PGI
	Phosphoglucomutase	2.7.5.1	PGM
	Tetrazolium oxidase	-	TO
Unsuitable			
	Acetaldehyde oxidase	1.2.1.3	AO
	Adenylate kinase	2.7.4.3	ADK
	Alcohol dehydrogenase	1.1.1.1	ADH
	Alkaline phosphatase	3.1.3.1	APH
	Fumerase	4.2.1.2	FUM
	α-Glycerophosphate dehydrogenase	1.1.1.8	αGPDH
	Malic dehydrogenase	1.1.1.37	MDH
	Xanthine dehydrogenase	1.2.3.3	XDH

TABLE 29

Relative Enzyme Mobility

	CELL LINE DESIGNATION	ENZYMES										
		EST	G6PDH	HK	IDH-1	IDH-2	LDH	LAP	ME	PGI	PGM	TO
1	ATC-10	73,98,134, 167	285	124	N	100,106,113	100,125	N	149	196	58	100,327
2	ATC-15	80	295	86,124	N	113	125	N	132	196	85	327
3	BTI-EAA	95	225	97	117	N	N	NT	121	115	90,98	NT
4	BM-N	22,83,100, 127	345	146	48	114	N	107	140	91,103,115	107	273
5	IPRI-MD-108	34,48,76, 171	225	115,136	50	95	80	100	130,137,146	67	71	209
6[a]	IPLB-LD-65Z	71	225	90	69	N	69	85	100	77	80	209
7	IMC-HZ-1	132	100	100	26	83	121	107	100	32,62,77	80	100
8[b]	IPLB-HZ-1075	102	100	100,115	100	100	100	100	100	100	100	100
9	IZD-MB-0503	102,122,135	225	90	69	N	80	85	108	67	85	209
10	IPLB-SF-21AE	100,115	100	100,115	100	100	100	100	100	100	100	100
11	UIV-SL-573	76,88,100, 115,135,185	110	90,128	100	113	114	100	100	100	89	273
12	TN-368	71	225	90	69	N	69	85	100	77	80	209
13	CP-1268	72	170	100	26	100	80	107	92	116	58	100
14	CP-169	115	170	115	18	100	100	128	92	100,109,116	58	209
15	MRRL-CH-I	80,132,145	100	115	26	106	53	96	132	136	85	300
16	IPRI-CF-124	78,132,145	170	132	36	100	94	N	149	37	96,100	B

Enzyme mobilities were recorded relative to the IPLB-SF-21AE enzyme patterns

B = blurred enzyme pattern
N = nil enzyme activity or no staining reaction observed
NT = enzyme not tested

a = Probably TN-368
b = Probably IPLB-SF-21AE

It is suggested that electrophoretic isozyme analyses may prove to be the most useful criterion for cell line identity and that the technique should become routine in laboratories where more than one invertebrate cell line is being handled.

Cellulose-acetate gel electrophoresis of invertebrate cell line isozymes (S.E. Brown and D.L. Knudson). The cellulose-acetate electrophoretic technique demonstrated that samples which were prepared and stored for several years at -70°C yielded resolvable isozyme phenotypes for the four isozymes tested. Further, these data demonstrated that the calculated relative mobilities were comparable with the values obtained using a starch gel electrophoretic system. Three of the sixteen samples exhibited a loss of enzymatic activity with storage. For example, the IMC-HZ-1 and IPRI-CF-124 stored samples did not exhibit ME activity, while BTI-EAA did not exhibit IDH and PGI activity. Freshly prepared ATC-15 and IPLB-SF-21AE exhibited the same isozyme activity and mobility as their stored counterparts. The enzyme activities lost in the prolonged storage of BTI-EAA, IMC-HZ-1, and IPRI-CF-124 were detected in samples prepared fresh.

Table 30 lists the relative mobilities of the cellular isozymes by electrophoretic separation on cellulose-acetate. This system utilizing the four isozymes was not able to distinguish IPLB-LD-65Z, IZD-MB-0503, and TN-368 from each other; or IPLB-SF-21AE from IPLB-HZ-1075.

A simple and reliable alternative to the starch gel system is the cellulose-acetate electrophoretic system. Since isozyme patterns have been demonstrated to be useful for distinghishing and identifying invertebrate cell lines, the simplified cellulose-acetate electrophoretic system may be the system of choice for routine screening of invertebrate cell lines.

A comparison of the ability of electrophoretic techniques utilizing the two types of electrophoretic media to separate cellular isozymes was made, and the results from the two electrophoretic systems were comparable. The inability to distinguish TN-368 from IPLB-LD-65Z and to distinguish IPLB-HZ-1075 from IPLB-SF-21AE was not probably due to the resolution of the cellulose-acetate or the starch gel system, but it is probably a result of a mishandling of cells. IZD-MB-0503 was identical to IPLB-LD-65Z and TN-368 using the four isozyme systems. The starch gel system was able to distinguish IZD-MB-0503 as a distinct cell line because the system gives better resolution in separating closely migrating bands.

The prolonged storage of samples affected only a few samples. Since BTI-EAA, IMC-HZ01 and IPRI-CF-124 cells prepared fresh demonstrated enzyme activity for all the enzymes tested, samples may be either prepared fresh or stored at -70°C. Although prolonged storage did result in a loss of enzyme activity for a few samples,

Table 30

Relative Enzyme Mobility

	CELL LINE DESIGNATION	ENZYMES			
		IDH	ME	PGI	PGM
1	ATC-10	162	204	253	54
2	ATC-15	162	165	253	87
3	BTI-EAA	115*	137	100*	100
4	BM-N	55, 162	176	114	110
5	IPRI-MD-108	70, 140	165	72	74
6	IPLB-LD-65Z	70	100	72	79
7	IMC-HZ-1	35,115	100*	63	79
8	IPLB-HZ-1075	100, 140	100	100	100
9	IZD-MB-0503	70	100	72	79
10	IPLB-SF-21AE	100, 140	100	100	100
11	UIV-SL-573	100, 162	100	100	92
12	TN-368	70, 162	100	72	79
13	CP-1268	35, 140	100	114	79
14	CF-169	24, 140	93	114	54
15	MRRL-CH-I	35, 145	165	157	87
16	IPRI-CF-124	35, 140	93*	58	100

Enzyme mobilities were recorded relative to the IPLB-SF-21AE enzyme patterns.

*Nil enzyme activity was found in stored samples, but enzyme activity was detected in fresh preparations.

it does not change the migration of most enzymes. Furthermore, freshly prepared samples exhibited the same isozyme patterns as the stored samples. Therefore, the fresh material may be compared to stored standards of most cell lines when identity checks of cell lines are made.

Cellulose-acetate electrophoresis of dipteran and acarine cell line isozymes (S.E. Brown and D.L. Knudson). The cell lines analyzed are listed in Table 31 , and Table 32 lists the relative mobilities of the four cellular isozymes. These data are indicative that the genera can be distinguished. However, there appear to be two distinct isozyme phenotypes for the Ae. albopictus cell lines. In the PGM staining system, AAAI, AAC7, and SAAR-C6/36 exhibit an additional band migrating at a value of 67. Cloned Ae. albopictus cell lines (DB-1 to 4) display phenotypes identical to their parent line, ATC-15. The cell line ATC-10 (new) was recently received at YARU, and it does not exhibit the Ae. aegypti isozyme phenotype. Since it is indistinguishable from the three Ae. albopictus cell lines, it may represent a mislabeled or contaminated cell line.

The use of electrophoretic analyses of cellular isozymes for the characterization of invertebrate cell lines is feasible. The technique is fast, it can be done economically in comparison to other less reliable methods, and it is ideally suited to the screening of a large number of samples.

Double-stranded RNA analyses of Orbiviruses (D.L. Knudson). Double-stranded (ds) RNA segment analyses of orbiviruses has revealed extensive genetic heterogeneity in field isolates. As reported previously (YARU Annual Report, 1978), forty-two isolates representing eighteen serotypes from the Kemerovo serogroup have been analyzed. Two serotypes, Nugget and Tribec, exhibited more than 10 distinct bands when their RNA segments were electrophoresed in polyacrylamide gels. Since orbiviruses are thought to be comprised of viruses which have 10 segments of RNA, these findings of additional bands were significant. Whether these isolates represent unusual viruses or they were the result of mixed virus infection remains to be demonstrated. Multiple clones of the two viruses have been prepared, and experiments are in progress to analyze this RNA segment profile of the cloned viruses, as well as their serologic properties.

Experiments are currently in progress to determine the dsRNA profiles for 15 members of the Changuinola serogroup and 6 members of the Palyam serogroup. Preliminary data indicate the cytoplasmic extract technique as described previously (YARU Annual Report, 1978) has not proven productive with these viruses. Modifications of the technique have been tested in an attempt to improve the consistency of producing an interpretable dsRNA profile.

Table 31

Cell lines analyzed by cellulose-acetate electrophoresis

Genus species	Cell line designation	Assigned number
Aedes aegypti	ATC-10	2
	ATC-10 (new)	3
Aedes albopictus	ATC-15	1
	SAAR-C6/36*	4
	AAAI**	5
	AAC7**	6
	DB-1*	7
	DB-2*	8
	DB-3*	9
	DB-4*	10
Aedes pseudoscutellaris	AP-61	11
Toxorhynchites amboinensis	PRU-TA42	12
Rhipicephalus appendiculatus	RA-243	13

* Cloned cell lines; DB-4 is persistently infected with Sindbis virus.

**Cell lines received from Dr. V. Stollar, Rutgers Univ. and one is currently thought to be the parent line to SAAR-C6/36.

Table 32

Relative Enzyme Mobility

CELL LINE DESIGNATION	ENZYMES			
	IDH	ME	PGI	PGM
1 ATC-15	162	165	253	87
2 ATC-10	162	204	253	54
3 ATC-10 (new)	162	165	253	67,87
4 SAAR-C6/36	162	165	253	67,87
5 AAAI	162	165	253	67,87
6 AAC7	162	165	253	67,87
7 DB-1	162	165	253	87
8 DB-2	162	165	253	87
9 DB-3	162	165	253	87
10 DB-4	162	165	253	87
11 AP-61	79,162	198	278	74
12 PRU-TA 42	162	198	232	37
13 RA-243	140	13	17	16

Enzyme mobilities were recorded relative to the IPLB-SF-21AE enzyme patterns.

Maintenance of Rhipicephalus appendiculatus cell cultures (LSTM-RA-243) by trypsinization (B.Q. Chen, M. Pudney, T.L.M. Coimbra, A.C. Steere, and S.M. Buckley). Beginning in 1971, developing adult tissues of Rhipicephalus appendiculatus were used by Drs. Varma and Pudney of the London School of Hygiene and Tropical Medicine to establish the first continuous tick cell lines (Varma, et al., J. Med. Ent., 11: 698, 1975). The cells were grown in glass bottles and subcultured by mechanical dispersion. However, in four attempts, investigators at Yale University were unable to maintain the cell lines by this method. Therefore, we attempted to subculture the particularly vigorous cell line, Rhipicephalus appendiculatus (LSTM-RA-243) by routine trypsinization, a method used frequently for maintenance of vertebrate cell lines.

Methods: Dr. Pudney supplied starter tick cell cultures (LSTM-RA-243) in the 323rd transfer. The following procedure was adapted in our laboratory. Fetal bovine serum, stored frozen in a Foster freezer at -20°C, was thawed rapidly at ambient temperature and then incubated at 37°C for 24 hours prior to incorporation into Leibovitz L-15 medium (10%/v) (Gibco, Grand Island, New York); additional inactivation by conventional heat treatment (56°C for 30 minutes) was omitted. Plastic flasks (25 cm^2, 75 cm^2, 150 cm^2, 490 cm^2, and 850 cm^2) (Corning Glass Works, Corning, New York) were filled with sterile, distilled, demineralized water for 2 days prior to transfers and incubated at 37°C (all volumes in the following narrative are calculated for 25 cm^2 flasks; appropriate amounts for other sized flasks are given in Table 33). One day prior to transfers, the distilled water was discarded and 3.5 ml of growth medium (Leibovitz L-15 medium supplemented with 10% tryptose phosphate broth, 10% fetal bovine serum, penicillin (100 u/ml) and streptomyinc (10 mcg/ml) (all GIBCO), and in some experiments, amphotericin B (10 mcg/ml) were added to cover the growth area. The flasks were then incubated at 37°C for 24 hours. On the day of transfers, the "spent" medium (i.e., the medium in which the tick cells had been growing) was pipetted into an Erlenmeyer flask and the "conditioned" medium (i.e., the medium incubated in new flasks at 37°C for 24 hours) was added to the "spent" medium in equal amounts (thereafter referred to as "half and half" medium).

Cultures with dense monolayers were preferably selected for transfers; they were rinsed with 5 ml of Puck's saline A (Puck et al., J. Exp. Med. 108: 945, 1958). After complete removal of the saline, washed monolayers were exposed briefly to 5 ml of 0.25% trypsin in Puck's saline A; and all of it, except for 0.2 ml, was quickly removed. The cells started to round up and detach from plastic immediately or within a few minutes depending on the density of the monolayer. The cells were then suspended in 2 ml of "half and half" medium. The resulting cell suspension was pipetted 20 to 60 times with a 2.0 ml pipette to produce a single cell suspension without damage to cells and diluted appropriately with additional

Table 33

Volumes of Materials According to Flask Size

Amounts of (ml)	Flask Size (cm^2)				
	25	75	150	490	850
Growth medium					
"conditioned"	3.5	10.5	21	25	50
"spent"	3.5	10.5	21	25	50
Puck's saline A	5	10	20	50	100
Trypsin (0.25%) in Puck's saline A	5	10	20	50	100
Remaining trypsin after initial use	0.2	0.5	1	3	5
"Half and half" medium	2	3	4	5	10
Cell suspension in "half and half" medium	7	21	42	50	100

"half and half" medium to effect a 1:2 or 2.3 split ratio. New flasks (parent flasks were not used) were then seeded with 7.0 ml of cell suspension, incubated at 28°C, and optimally, not examined during the following 72 hours to insure good attachment of cells to plastic. The 2 tissue culture roller flasks, 490 cm^2 and 850 cm^2, were placed on a tissue culture roller apparatus at one rotation every 3 minutes.

Results: At the time of this writing, tick cell cultures (LSTM-RA-243) have been trypsin-dispersed at 6 to 15 day intervals more than 12 times (Table 34). By enzymatic disaggregation, the cells were dispersed uniformly, attached readily to plastic, and grew to confluence in 6 to 15 days. The cell line tolerated the routine addition of penicillin and streptomycin, but did not allow the continuous incorporation of amphotericin B. The technical procedures were done successfully by three different investigators and two assistants in research.

In brief, we believe that the tick cell line LSTM-RA-243 can be subcultured more easily by trypsinization than by mechanical dispersion. This method may encourage other investigators to use this cell line in the study of tick-borne infectious agents.

Cold preservation of the Rhipicephalus appendiculatus (LSTM-RA-243)cell line (B.Q. Chen and S.M. Buckley). Cells from confluent Rhipicephalus appendiculatus cultures were dispersed with trypsin and resuspended in a medium consisting of "spent" medium (i.e., the medium in which the tick cells had been growing) (89%), dimethylsulfoxide (10%), and penicillin-streptomycin mixture (1%) (penicillin 100 units/ml; streptomycin 100 mcg/ml). The remainder of the "spent" medium was saved. Cells in storing medium were dispensed in 1.0 ml amounts into pro-vials which were immediately placed in cotton wool in an ice cream box made of expanded polystyrene. The box was then stored in a Revco freezer at -65°C for at least 16 hours, after which the pro-vials were quickly transferred to a Linde liquid-nitrogen refrigerator.

Two days prior to recovery of cells, plastic flasks (25 cm^2) were filled with sterile, demineralized distilled water and incubated at 37°C for 24 hours. One day prior to recovery of cells, the distilled water was discarded and 2.0 ml of growth medium [Leibovitz L-15 medium supplemented with 10% tryptose phosphate broth, 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 mcg/ml)] were added to cover the growth area. The flasks were then incubated at 37°C for 24 hours. For recovery of viable cells, each pro-vial was thawed rapidly at 37°C, and the contents were withdrawn with a Pasteur pipette, added to 5.0 ml of "spent" medium, and centrifuged at 1500 rpm for 10 minutes. The cell sediment was then resuspended in 5.0 ml of "spent" medium and 2.0 ml of "conditioned" medium (i.e., medium incubated in new flasks at 37°C for 24 hours) and incubated at 28°C in the pre-treated 25 cm^2 flasks. Recovery of viable Rhipicephalus appendiculatus (LSTM-RA-243) cells was successful in two separate experiments.

Table 34

Flow Chart of Tick Cell Line (LSTM-RA-243) Transfers

Experiment	Date (1979)	Transfer Interval (Days)	Transfer Number	Transferred from	Transferred to	Split ratio
I	7/31	11	324	1* - 25 cm^2†	2 - 25 cm^2	1:2
	8/10	10	325	2 - 25 cm^2	4 - 25 cm^2	1:2
	8/17	7	326	4 - 25 cm^2	8 - 25 cm^2	1:2
	8/29	12	327	8 - 25 cm^2	16 - 25 cm^2	1:2
	9/4	6	328	10 - 25 cm^2	5 - 75 cm^2	2:3
	9/11	7	329	3 - 25 cm^2	1 - 490 cm^2	1:2.1
II	9/7	9	328	3 - 25 cm^2	1 - 150 cm^2	1:2
	9/14	7	329	3 - 150 cm^2	1 - 150 cm^2	1:2
III	8/29	12	327	8 - 25 cm^2	16 - 25 cm^2	1:2
	9/13	15	328	16 - 25 cm^2	32 - 25 cm^2	1:2
	9/21	9	329	3 - 25 cm^2	1 - 150 cm^2	1:2
	9/28	7	330	1 - 150 cm^2	2 - 150 cm^2	1:2

*Indicates the number of flasks

†Indicates the size of the growth area

High titered virus in the Igarashi clone of Singh's Aedes albopictus mosquito cell line (C6/36) inapparently and persistently infected with JE attenuated virus, 2-8 strain (B.Q. Chen and S.M. Buckley). The 2-8 vaccine strain of JE virus was developed in Peking by Chen (Acta Microbiologica Sinica 14(?): 176-184, 1974) from the SA 14 strain isolated in primary hamster kidney tissue culture, a topotype strain isolated in north-western China. The 2-8 vaccine strain was derived by 100 passages in primary hamster kidney tissue culture, followed by 2 treatments with ultraviolet light, 5 subcutaneous passages in baby mice, and 3 plaque cloning passages in primary chick embryo tissue culture. It is of reduced virulence for mice, pigs, horses, and monkeys. It has been administered experimentally to 8,000 children in China with approximately 50% seroconversion as ascertained by a standard neutralization test, i.e., using adult mice and the i.c. route of inoculation. The 2-8 vaccine strain is currently employed successfully as a live virus vaccine for horses in China. More than 500,000 horses have been vaccinated.

Because the infectivity titer of the 2-8 vaccine strain of virus is relatively low, especially when produced in large batches in primary hamster kidney tissue culture, attempts were made at Yale to improve the yield by use of Igarashi's clone C6/36 of Singh's Aedes albopictus cells (Igarashi, A.J., Gen. Virol. 40: 531-544, 1978). In addition to infectivity, HA and CF antigen production was measured, and the yields compared with those of other mosquito and of vertebrate cell lines.

The C6/36 cell line was kindly supplied by Dr. A. Igarashi, Japan, and was used between YARU passages 25 and 27. Cells were grown in 75 cm^2 or 150 cm^2 plastic tissue culture flasks incubated at 28°C. The growth medium was Leibovitz-15 (L-15) supplemented with 10% fetal bovine serum (or calf serum) and 10% tryptose phosphate broth. For maintenance, the serum was reduced from 10% to 3%, either fetal bovine serum or calf serum.

Cells were inoculated with a large dose of virus, i.e., undiluted fluid of a BHK-21 virus stock of the 2-8 vaccine strain. After an adsorption period of 1 hour at 28°C, the virus was removed, the cells were washed with Puck's saline A and an appropriate amount of maintenance medium was added. Subsequently, 1.0 ml samples were removed at intervals and tested for presence of virus. Comparative inoculations were made also in vertebrate cells incubated at 37°C. The titrations of virus were carried out in BHK-21 cells and the TCD_{50} was calculated according to the Reed-Muench method.

Table 35 compares results in Vero and C6/36 cells. When Vero cells exhibited a cytopathic effect (CPE) of 4+, i.e., 90-100% of cells detached from plastic, the cells were presumably killed and were no longer able to produce either CF antigen or infective virus. In the C6/36 cell line, CF antigen and virus were produced continually even after 4 days of 4+ CPE.

Table 35

The relation between CPE, TCD_{50} and CF antigen in Vero and C6/36 cell lines after inoculation with the 2-8 vaccine strain of JE virus

Cell line	Test	Days after inoculation								
		1	2	3	4	5	6	7	8	9
	CPE*	0	0	0	0	1+	2+	2+	4+	4+
Vero + JE virus	$log_{10}TCD_{50}/ml$	2.5	5.0	5.0	6.5	7.0	7.0	7.0	4.5	4.0
	CF titer	0	0	1/2	1/8	1/16		1/4	0	0
Virus control 37°C	$log_{10}TCD_{50}/ml$	4.5	3.0	3.0	3.0	2.5				
	CPE*	0	0	0	0	1+	4+	4+	4+	4+
C6/36 + JE virus	$log_{10}TCD_{50}/ml$	3.0	5.5	7.5	8.0	8.0	8.0			8.0
	CF titer	0	0	0	1/4	1/12	1/3			1/3
Virus control 28°C	$log_{10}TCD_{50}/ml$	5.0	4.0	3.5	2.5					

*CPE = denoting cellular detachment from plastic on a scale of 1+ to 4+

Most of the C6/36 cells were detached from the walls of plastic flasks 9 days after inoculation. The combined cell and fluid phase was collected and centrifuged, and the cell sediment was supplied with fresh medium and then introduced again into the same plastic flask used for primary infection of cells. As shown in Figure 5 , the virus was released rapidly; only 24 hours after adding fresh medium the titer was 8.0 $\log_{10}$ TCD_{50}/ml. This high titer was maintained for 47 days, provided the medium was changed at weekly intervals. In the reseeded flask, cells remained attached to plastic and CPE was not re-occurring.

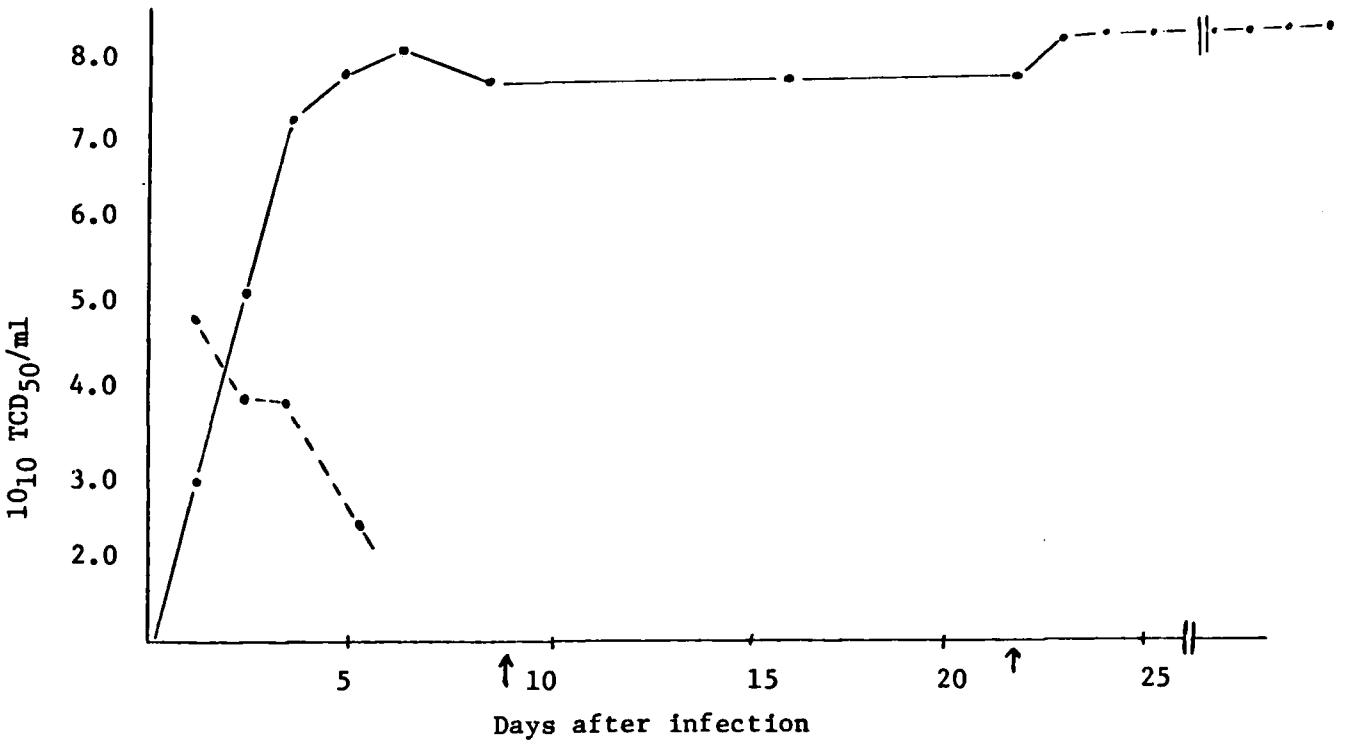
After persistent infection of 47 days duration, the old medium was removed completely and replaced with fresh medium every 24 hours, and the fluid phase was assayed for presence of virus, and for HA and CF antigens. As seen in Figure 6 , the 24 hour yield was 8.0 $\log_{10}$ TCD_{50}/ml.

CF antigen is produced by the C6/36 cells both during the primary and the persistent infection, although the yield is different in the two phases. During primary infection, the highest CF titer was obtained 5 days after inoculation (Fig. 7), and then it dropped quickly; in persistent infection, a relatively high titer was maintained for at least 21 days after inoculation, provided the medium was changed at weekly intervals. When the medium was replaced at 24 hour intervals, 47 days after primary infection of cells, the titer of CF antigen was not as high initially, but the culture still produced CF antigen in each 24 hour harvest. By contrast, HA antigen still titered 1:4 to 1:8 at 24 hour intervals, and 47 days after primary infection.

A growth curve was carried out 21 days after the initiation of persistent infecton of C6/36 cells with the 2-8 vaccine strain of JE virus. The old medium was completely replaced with new medium. Subsequently, 1.0 ml aliquots of fluid phase was removed at 1, 3, 6, 8, and 24 hours and assayed for the presence of virus, HA, and CF antigen. As seen in Fig. 8 , the infectivity titer rose very quickly. There was no latent period. In the 1 hour sample, the TCD_{50} titer was 6 $\log_{10}$/ml, and in the 8 hour sample 8.0 $\log_{10}$/ml. The HA and CF antigens also appeared quickly. In the 24 hour sample, the titers of HA, and CF antigens were 1:16 and 1:4, respectivley.

Thus, the C6/36 cells persistently infected with the 2-8 vaccine strain of JE virus produce large amounts of virus over several days provided the medium is changed at weekly intervals. Immediately following the change of medium, infective virus is released and within 8 hours, the persistently infected cells produce as much as 8 $\log_{10}$ TCD_{50}/ml. Thus, this cell system offers the potential for large scale JE vaccine production, inexpensively and on a continuous basis.

Fig. 5 Persistent infection of the Igarashi clone C6/36 of Singh's Aedes albopictus cells with the vaccine strain 2-8 of JE virus.

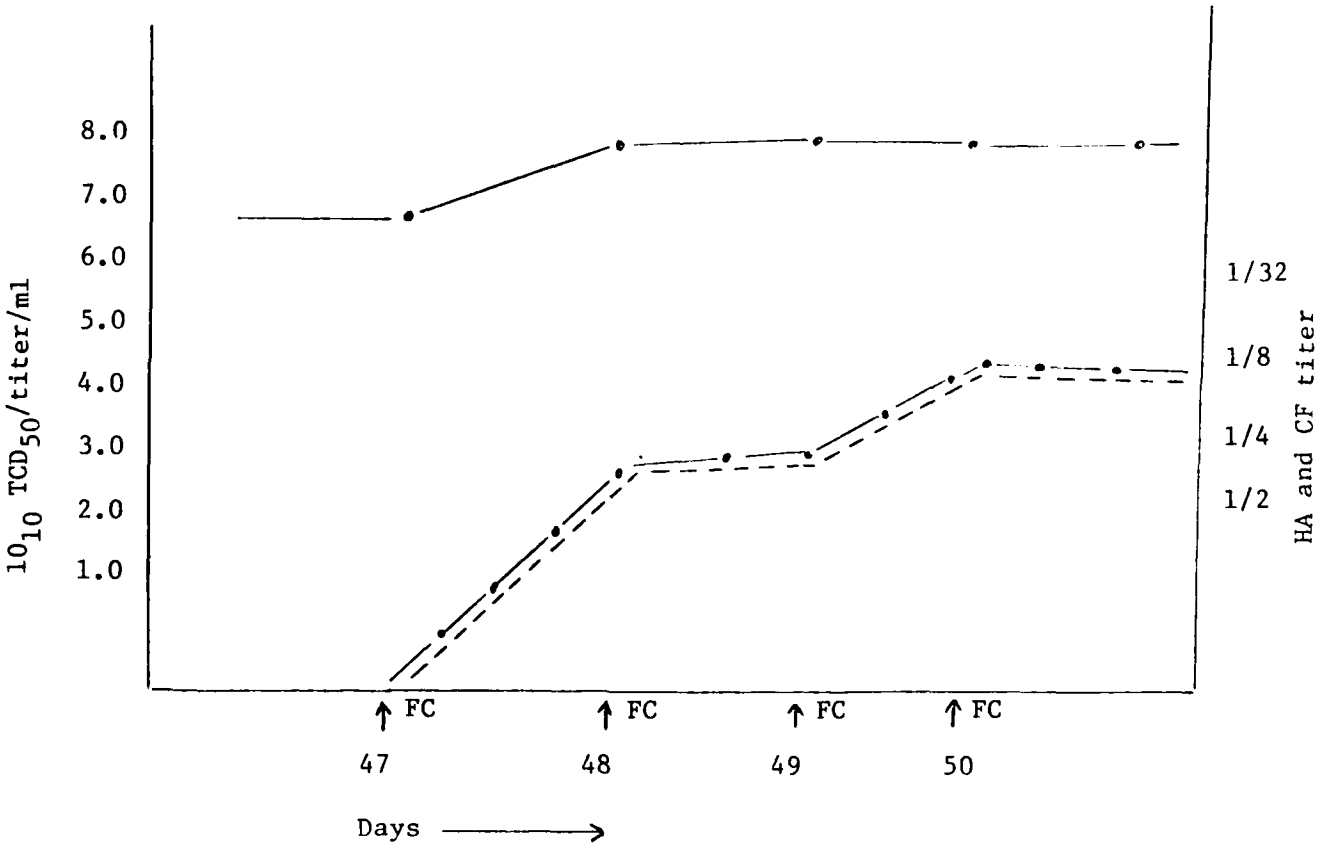


•————• Infectivity titer expressed as $\log_{10}$ TCD_{50}/ml

•---------• Virus control (without cells) at 28°C

↑ Arrow denotes change of fluid phase

Fig. 6 Infectivity titers, complement-fixation titers and hemagglutinin titers obtained in the Irarashi clone C6/36 of Singh's Aedes albopictus cells persistently infected with the vaccine strain 2-8 of JE virus.



Infectivity titer

Complement-fixation titer

Hemagglutinin titer

↑ FC: Indicating changing of the fluid phase at 24 hour intervals

Fig. 7 The yield of complement-fixation antigen in Igarashi's clone C6/36 of Singh's Aedes albopictus cells infected with the vaccine strain 2-8 of JE virus.

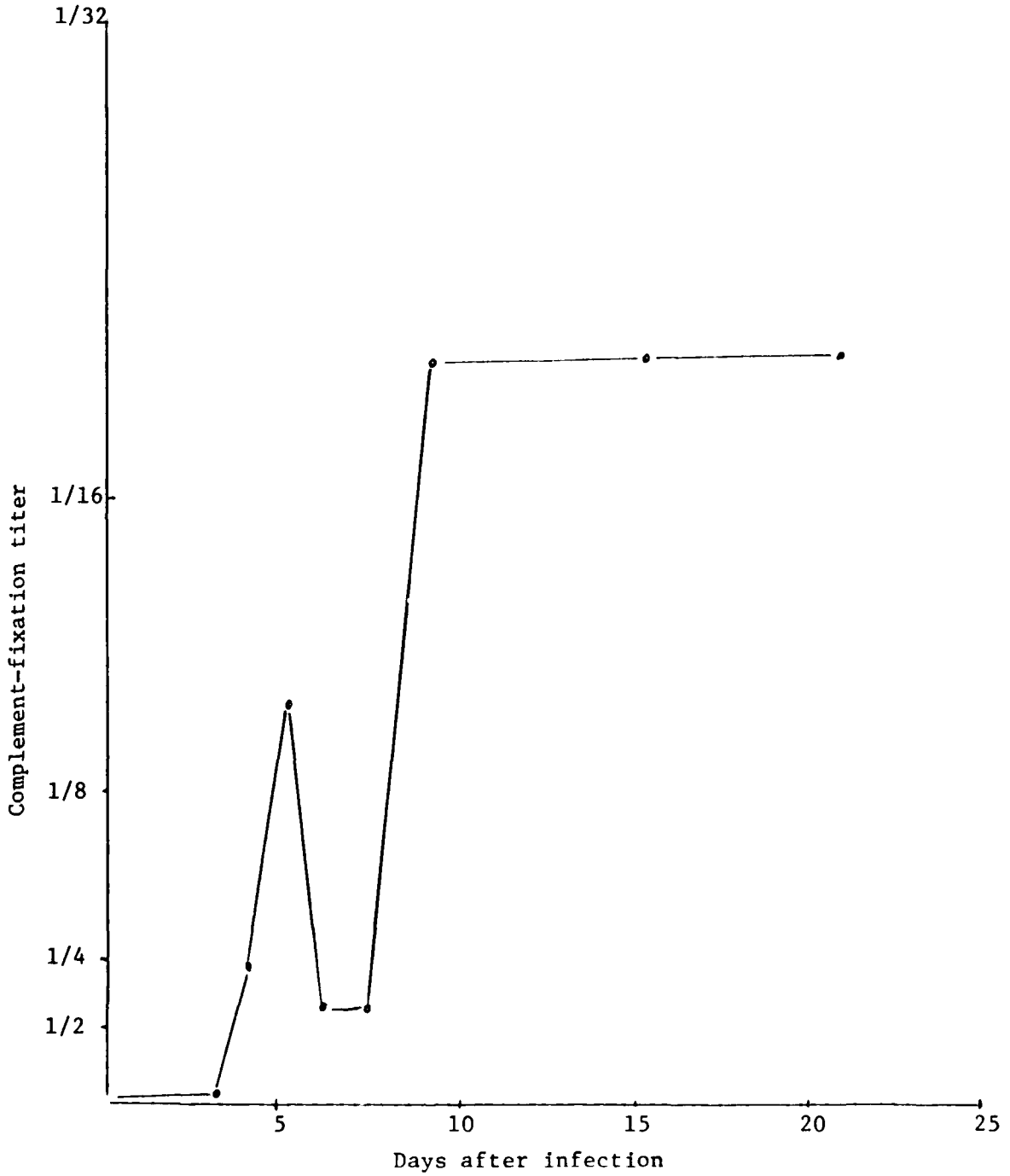
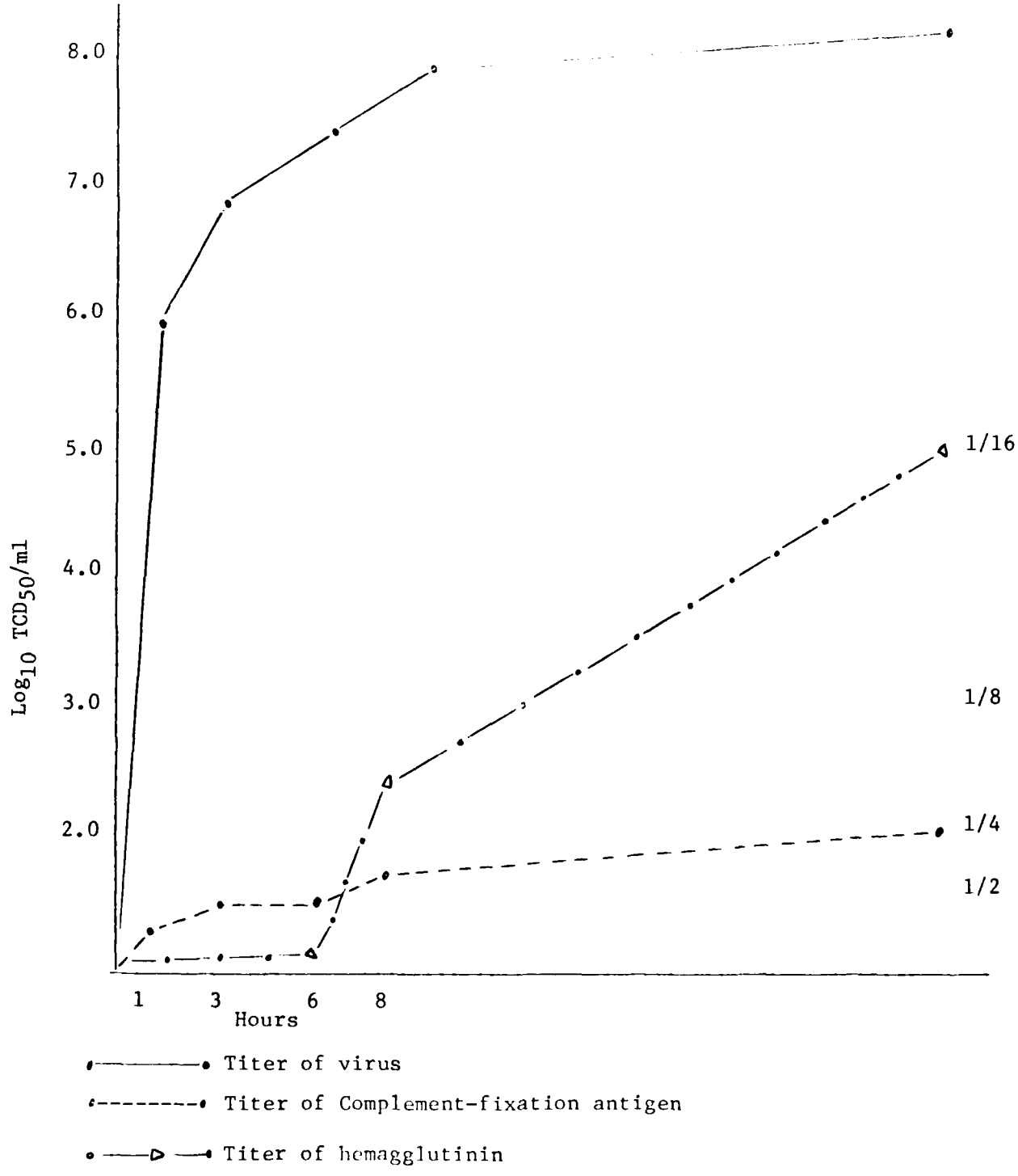


Fig. 8 Infectivity titers, titers of complement-fixation antigens, and titers of hemagglutinin at different intervals (hours) after change of the fluid phase of C6/36 cells persistently infected with the vaccine strain 2-8 of JE virus



The enzyme linked immunosorbent assay (O. Wood and C. Frazier). The ELISA test was used to detect antibody in rabbit sera. Antigen was prepared by PEG precipitation and sucrose gradient centrifugation of infected Vero cell culture fluid. The test was carried out without elaborate apparatus in a manner fully applicable to use under field conditions. The plates were coated with antigen and washed; sera were added then the conjugate and substrate. Alkaline phosphatase conjugate was used with p-nitrophenyl phosphate as substrate. The test was read by eye.

Results (reported in part last year) of ELISA of Bunyamwera group viruses are shown in Table 36. Bunyamwera, Germiston, Batai, and Ilesha were closely related (ratio of homologous to heterologous titer 2 to 16) and had minimal cross-reactivity with Sororoca, Wyeomyia and Guaroa viruses (Ho/H+ = 256 to 10240). Birao showed a slight cross-reactivity with Bunyamwera, Batai, Germiston, and Ilesha and a stronger cross-reaction with Cache Valley virus. The Cache Valley serum was broadly cross-reactive. Wyeomyia and Sororoca cross-reacted inter se but not greatly with other members of the Bunyamwera group.

Oriboca was included as a control bunyavirus, not in the Bunyamwera group. Interestingly, it reacted slightly with Cache Valley and Guaroa indicating the high sensitivity of the ELISA. The ELISA detected the same inter-relationships among Bunyamwera group viruses as did HI and plaque reduction neutralization tests reported in published literature.

To establish the relative sensitivity of the ELISA test and to determine if it can be used in human serum surveys, 60 African human sera were tested with Bunyamwera, Birao, Batai, Germiston, Ilesha, and Guaroa antigens. HI tests were done concurrently. The results are shown in Table 37. Correlation was excellent if one assumes that ELISA is more sensitive than HI. All sera positive by HI were also positive by ELISA. Some sera were positive by ELISA and negative by HI; in most cases these were low-titered ELISA positives.

The ELISA test was applied to the study of cross-reactions in the California group. Antigens were prepared in BHK-21 cells and tested with mouse ascitic fluids and an anti-mouse conjugate. The results are shown in Table 38. These results correspond very closely in titer and specificity to results of plaque reduction neutralization tests performed with the same fluids. Two fluids showed non-specificity in the 1:10 and 1:20 dilutions.

Comparative tests of sera of 48 persons resident in Connecticut by ELISA and HI revealed one strongly positive by both tests for LaCrosse virus and another strongly positive by both tests for

Table 36

ELISA of Bunyamwera group whole virion antigens

Antigens	Rabbit sera, 3 injections										Control Normal
	BUN	GER	BIR	BAT	ILE	CV	SOR	WYO	GRO	Control ORI	
[illegible]	10,240[a]	40	320	1280	1280	320	<40	80	320	40	0
[illegible]ER	1280	2560	320	1280	2560	320	0	0	0	0	0
[illegible]IR	320	40	1280	0	320	40	0	0	40	40	0
[illegible]AT	5120	160	640	10,240	2560	320	0	40	160	0	0
[illegible]LE	5120	320	1280	640	10,240	640	0	0	320	20	0
[illegible]V	640	0	160	80	320	1280	0	40	80	40	0
[illegible]R	40	0	0	0	0	40	1280	320	40	0	0
[illegible]O	0	0	0	0	0	0	<80	80	80	0	0
[illegible]RO	0	0	>320	0	0	0	0	0	320	0	0
[illegible]RI	0	0	0	0	0	0	0	0	0	80	0

[illegible] = <20.

Table 37

Results of ELISA and HI tests with Bunyamwera group antigens and Ethiopian human sera

Serum No.	Bunyamwera		Batai		Germiston		Ilesha		Birao	Guaroa	Cache Valley
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	ELISA	HI
1	20	0	20	0	10	0	20	0	10	10	0
2	160	40	80	0	0	0	20	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0
4	20	10	20	20	640	20	20	0	0	0	0
5	>40	20	20	0	0	0	>40	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0
7	640	20	20	0	20	0	20	0	10	0	0
8	0	0	0	0	0	0	0	0	0	0	0
9	10	0	10	0	0	0	0	0	0	0	20
10	0	0	0	0	0	0	0	0	0	0	0
11	320	20	20	0	10	0	10	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0
14	40	0	0	0	0	0	10	0	0	0	0
15	320	20	320	40	20	10	640	40	0	0	20
16	0	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0	0
18	640	0	320	0	0	0	0	0	0	0	0
19	40	0	40	0	20	0	20	0	>40	0	0
20	20	0	640	0	10	0	0	0	10	0	20
21	0	0	0	0	0	0	0	0	0	0	10
22	0	0	0	0	0	0	0	0	0	0	0
23	80	10	160	20	160	20	80	40	0	0	20
24	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0
27	0	0	20	0	0	0	20	0	0	0	10
28	0	0	0	0	0	0	0	0	0	0	0
29	20	0	0	0	0	0	0	0	0	0	0
30	160	0	0	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0
32	20	0	20	0	10	0	10	0	0	0	0
33	0	0	40	0	0	0	20	0	0	0	0
34	20	20	20	10	640	10	20	40	0	0	0
35	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0	0
37	640	0	20	0	640	10	20	0	0	0	0
38	0	0	80	0	0	0	40	0	0	0	0
39	0	0	0	0	0	0	0	0	0	0	0
40	20	20	160	40	10	40	20	0	0	0	20
41	20	10	10	0	10	10	160	40	0	0	0
42	40	10	80	40	80	10	10	0	0	0	20
43	0	0	0	0	0	0	0	0	0	0	10
44	20	20	10	0	10	0	10	0	0	0	0
45	40	10	640	0	640	40	160	0	0	0	20

cont.

Table 37 contd.

Results of ELISA and HI tests with Bunyamwera group antigens and Ethiopian human sera

Serum No.	Bunyamwera		Batai		Germiston		Ilesha		Birao	Guaroa	Cache Valley
	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	ELISA	HI
46	0	0	40	10	0	0	40	0	0	0	0
47	0	0	0	0	0	0	0	0	0	0	0
48	0	0	40	10	160	0	40	0	0	0	0
49	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0
51	160	10	40	10	20	20	640	0	0	0	10
52	0	0	0	0	0	0	0	0	0	0	0
53	0	0	0	0	0	0	0	0	0	0	0
54	20	10	20	0	0	0	40	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0	0	0
58	0	0	0	0	0	0	0	0	0	0	0
59	0	0	0	0	0	0	0	0	0	0	0
60	20	0	20	0	0	0	640	0	0	0	0

Table 38

California group cross-reactivity by ELISA

Antigen	Antibody: La Crosse	Snowshoe hare	Trivittatus	Tahyna	Jamestown Canyon	Guaroa	Control DEN-1	Control Normal
La Crosse	2560	640	0	40	20	10	0	0
Snowshoe hare	320	2560	80	320	640	40	0	0
Trivittatus	10	80	640	20	320	10	0	0
Tahyna	160	320	0	640	320	10	0	0
Jamestown Canyon	80	160	20	10	2560	0	0	0
Guaroa	0	80	10	40	20	320	0	0
Control DEN-1	0	0	10	20	0	0	1280	0

Jamestown Canyon virus. The LaCrosse positive was confirmed by plaque reduction neutralization test.

ELISA antigens for dengue types 1-4 and West Nile were prepared in LLC-MK2 or Vero cells. The results of tests with mouse ascitic fluids are shown in Table 39. There was notable lack of specificity and it must be concluded that the ELISA, at least under the conditions of these tests, offers no advantage over the HI test for flaviviruses.

Experiments were initiated to determine the feasibility of using ELISA to detect antigen in human sera and in mosquito pools. The coating antibody was prepared from LaCrosse mouse ascitic fluid by ammonium sulfate precipitation and Sephadex G-200 column chromatography. The unknown sample was diluted in PBS and reacted in the coated well. After washing the well, rabbit anti LaCrosse serum followed by alkaline phosphatase anti-rabbit conjugate and substrate were added.

Approximately 10^5 LD_{50}/ml of La Crosse virus could be detected readily when the virus was artificially added to the following:

Aedes aegypti	pools of 10 and 50
Aedes triseriatus	pools of 10 and 50
Culiseta melanura	pools of 10 and 50
Culex fatigans	pools of 10 and 50
Culex restuans	pool of 21
mouse serum 20%	
mouse liver 20%	
mouse brain 20%	
mouse brain sucrose-acetone antigen	
Vero tissue culture fluid	
human CSF	
LLC-MK2 dengue ELISA antigen	

None of the above were positive when used as control preparations diluted in PBS. Approximately 1 in 25 human sera were positive non-specifically. The reason for the non-specificity is not known.

Table 39

ELISA of dengue and West Nile viruses

	Antibody							
Antigen	DEN-1	DEN-2	DEN-3	DEN-4	WN	ILESHA Control	Normal	PBS
Den 1	160	40	640	0	320	0	0	0
Den 2	320	320	640	160	320	0	0	0
Den 3	40	20	640	40	320	0	0	0
Den 4	40	20	40	80	40	0	0	0
WN	20	0	640	40	2560	0	0	0
Ilesha	0	20	20	20	20	160	0	0

0 = <10

VI. Distribution of reagents, WHO Collaborating Centre for Reference and Research (R. Shope, J. Casals, A. Main, O. Wood, S. Buckley, G. Tignor). The equivalent of 518 ampoules of arbovirus reagents were distributed from the WHO Centre to laboratories in 17 countries during the period 1 January 1979 to 31 December 1979. This total consisted of 228 ampoules of virus stock, 62 ampoules of virus antigen, and 228 ampoules of mouse immune ascitic fluid or sera. Of the viruses and antibody distributed, this represented 164 different arboviruses.

During 1979, the equivalent of 398 ampoules of arbovirus reagents were referred to this Centre from laboratories in 16 different countries. The referrals consisted of 54 ampoules of virus specimens (Table 40), 288 ampoules of virus antigens, and 56 ampoules of immune reagents. In addition, 374 sera were received for arbovirus antibody survey testing.

Eight different cell lines were distributed during 1979. The lines and recipients are listed in Table 41.

Table 40

Viruses referred to YARU for identification, 1979

Country of origin; strain	Source	Information from donor	YARU identification
Australia			
Douglas virus 93-6	Cow blood	Simbu group	Simbu group, probably new
CSIRO 153	Culicoides brevitarsis	Simbu group	Simbu group, probably new
Brazil			
76 V25880	Culex (Melanoconion) sp.	Same as 78 V213	Same as 78 V213
78 V213	Culex (Melanoconion) sp.	Ungrouped	Ungrouped
H 350698	Man	yellow fever	
Ar 232869	Haemagogus sp.	yellow fever	
Ar 350397	Haemagogus sp.	yellow fever	
Czechoslovakia			
Argas 265	Argas persicus	picornavirus	
France			
Brest Ar T101	Ornithodorus maritimus	Soldado-related	Soldado-related
Gambia			
79 V1463	Anopheles spp.	Unidentified	Tataguine-related
Germany			
HH, KF, MW, WH, RS, MD, HT, and KD	Human CSF	Amyotrophic lateral Sclerosis agents (?)	

Cont.

Table 40

Viruses referred to YARU for identification, 1979

Country of origin; strain	Source	Information from donor	YARU identification
India			
77-2366-17	Ornithodoros piroformis	unidentified	
Italy			
ISS Phl 3	Phlebotomus pappataci	New virus, phlebotomus fever group	
Senegal			
Dak An D512	Tatera (gerbil)	Strain of Gordil virus	
Uganda			
SG 38485	Man	Same as Z 52963	
SG 38594	Man	Same as Z 52963	
Amp 15023	Amblyomma variegatum	Ungrouped	
Z 52963 (Bat 983)	Rousettas aegyptiacus	Ungrouped	
Z 52969 (Bat 989)	Rousettas aegyptiacus	Same as Z 52963	
SG 38495	Man	Same as Z 52963	
Amp 15332	Amblyomma variegatum	Ungrouped	
SG 38545	Man	Same as Z 52963	

cont.

Cont.

Table 40

Viruses referred to YARU for identification, 1979

Country of origin; strain	Source	Information from donor	YARU identification
United States			
Bandia		For confirmation of identity	Bandia
Qalyub		For confirmation of identity	Qalyub
Hazara		For confirmation of identity	Hazara
Punta Toro		For confirmation of identity	
Karimabad		For confirmation of identity	
Y 62-33		For confirmation of identity	
CTF (Florio)		-	

Table 41

Vertebrate and invertebrate cell lines distributed during 1979

Name of recipient	Location	Date sent	Cell line	Number and Size of flask	
Dr. Robert Florkiewicz	The University of Arizona, Tucson, Arizona	4/4/79	Singh's *Aedes albopictus* ATC-15	2	75 cm^2
Dr. Robert Florkiewicz	The University of Arizona, Tucson, Arizona	5/14/79	Singh's *Aedes albopictus* ATC-15	2	75 cm^2
Dr. Jerome Kern	The American Type Culture Collection Rockville, Md.	6/8/79	Igarashi's clone C6/36 of Singh's *Aedes albopictus* ATC-15	2	25 cm^2
Dr. T.G. Ksiazek, Capt. USAF, VC	Jakarta Detachment NAMRU-II	6/8/79	Vero	2	25 cm^2
			BHK-21, clone 13	2	25 cm^2
Dr. E.A. Tkachenko	Institute of Poliomyelitis and Viral Encephalitides, Moscow, USSR	7/5/79	Vero	1	25 cm^2
			BHK-21, clone 13	1	25 cm^2
Dr. S.P. Chunikhin	Institute of Poliomyelitis and Viral Encephalitides, Moscow, USSR	7/5/79	*Aedes pseudoscutellaris*, MOS-61	1	25 cm^2
			Igarashi's clone C6/36 of Singh's *Aedes albopictus* cell line, ATC-15	1	25 cm^2
			Rhipicephalus appendiculatus LSTM-RA-219	1	25 cm^2
			Xenopus laevis LSTM-XTC-2	1	25 cm^2

Table 41 continued

Name of recipient	Location	Date sent	Cell line	Number and Size of flask	
Dr. P. Spath	University of Bern, Institute of Hygiene and Medical Microbiology, Switzerland	8/1/79	Igarashi's clone C6/36 of Singh's *Aedes albopictus* ATC-15	1	25 cm^2
			Singh's *Aedes albopictus* ATC-15	1	25 cm^2
			Aedes pseudoscutellaris LSTM-MOS-61	1	25 cm^2
Dr. J. Thorsen	Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario	9/10/79	*Aedes pseudoscutellaris* LSTM-MOS-61	1	25 cm^2
			Igarashi's clone C6/36 of Singh's *Aedes albopictus* ATC-15	1	25 cm^2
			Rhipicephalus appendiculatus LSTM-RA-243	1	25 cm^2
			Xenopus laevis LSTM-XTC-2	1	25 cm^2
Wuhan Institute of Virology, China c/o Prof. Kao Shang, through courtesy of Dr. Jowell Chao, Beverly Hills, Calif.	Wuhan Institute of Virology, China	9/10/79	Singh's *Aedes albopictus* ATC-15	1	25 cm^2
			Igarashi's clone C6/36 of Singh's *Aedes albopictus* ATC-15	1	25 cm^2
			Rhipicephalus appendiculatus LSTM-RA-243	1	25 cm^2
			Xenopus laevis LSTM-XTC-2	1	25 cm^2

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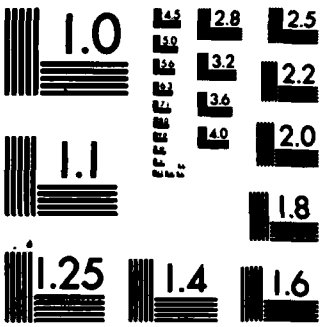

Table 41 continued

Name of recipient	Location	Date sent	Cell line	Number and size of flask	
Dr. Michael Reese	Dept. of Microbiology Southern Illinois University, Carbondale, Ill.	12/11/79	Singh's Aedes albopictus ATC-15	1	25 cm^2
			Igarashi's clone C6/36 of Singh's Aedes albopictus ATC-15	1	25 cm^2
Dr. Otavio Oliva c/o Dr. Francisco Pinheiro	Instituto Evandro Chagas Belem, Brazil	12/17/79	$LLC\text{-}MK_2$	1	25 cm^2
			BHK-21, clone 13	1	25 cm^2
			Vero	1	25 cm^2
			Igarashi's clone of C6/36 cells, of Singh's Aedes albopictus, ATC-15	1	25 cm^2
			Singh's Aedes albopictus, ATC-15	1	25 cm^2

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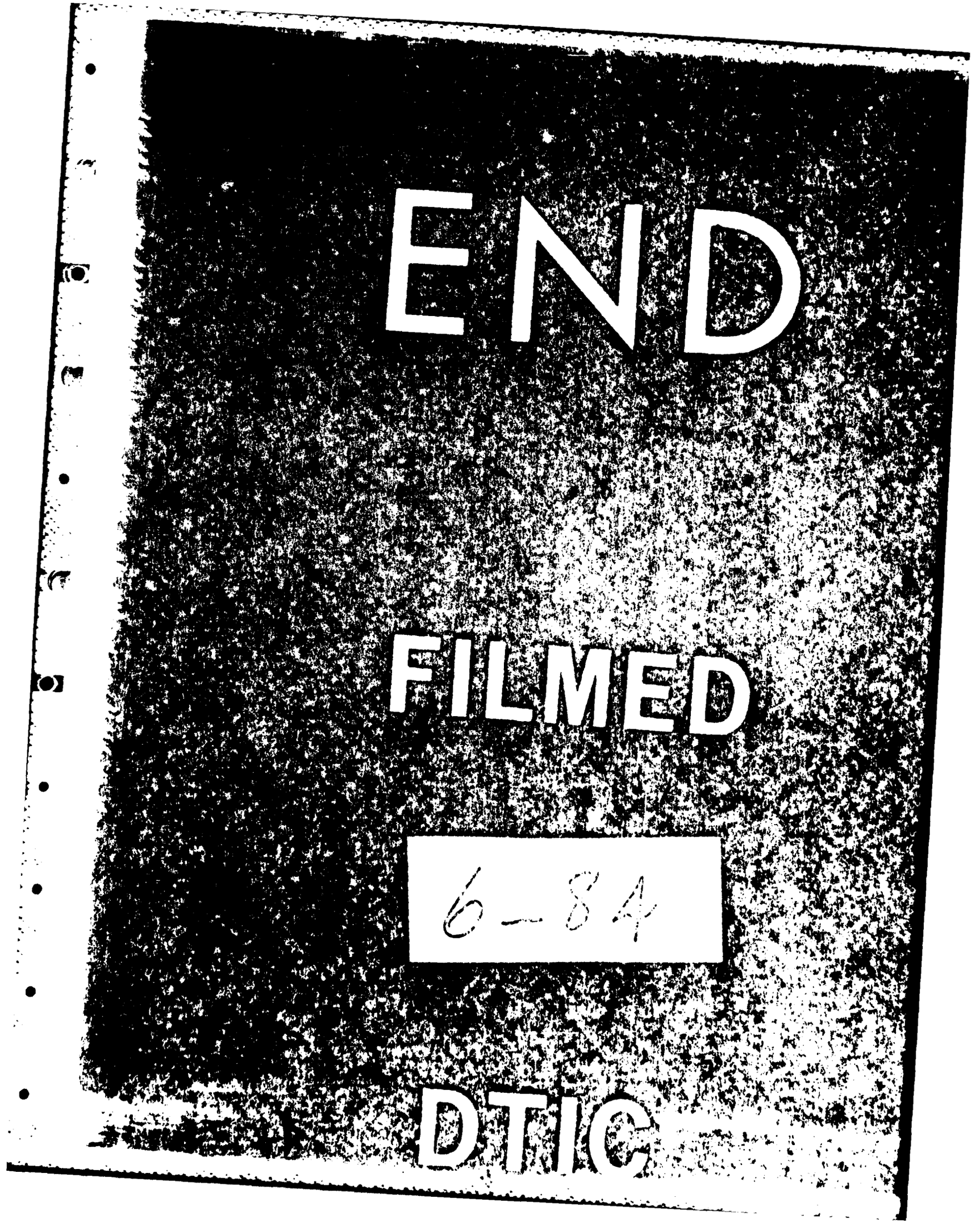
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